STRUCTURAL STUDIES OF SOME NATURAL POLYSACCHARIDES

A THESIS

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By

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CERTIFICATE

Certified that the thesis entitled, "STRUCTURAL STUDIES OF SOME NATURAL POLYSACCHARIDES" submitted by (Km.) Kahema Rani Gupta in fulfilment of the requirements for the Ph.D. degree of Bundelkhand University, embodies the record of her own research work, carried out under my supervision and guidance. She has worked regularly more than 240 days under the S.C.S. & T. Scheme, at the laboratory of Chemistry Department, Dayanand Vedic Post-graduate College, Orai of Bundelkhand University, JHANSI. (U.P.).

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PREFACE

The dissertation entitled, "STRUCTURAL STUDIES OF SOME NATURAL POLYSACCHARIDES", deals with the isolation and chemical examination of polysaccharides from the seeds of *Zizyphus jujuba* and *Phaseolus mungo*, some constituents from the seeds of *Daucus carota* and free water soluble monosaccharides from the flowers of *Linum usitatissimum*. The thesis has been divided in to five chapters.

The Chapter I is of introductory nature and describes the wide importance of natural products and a brief account of different classes of compounds, i.e. polysaccharides, sterols, flavonoides and free sugars.

The Chapter II deals with the isolation and structural elucidation of neutral water soluble polysaccharide from the seeds of *Zizyphus jujuba*.

The Chapter III describes the isolation and structural elucidation of a water soluble neutral polysaccharide from the seeds of *Phaseolus mungo*.

The Chapter IV, is divided into three Sections (A), (B) and (C), deals with the isolation and elucidation of chemical structures of a sterol and two flavonoids from the seeds of *Daucus carota*.

The Chapter V and the last chapter forms the subject matter of chemical examination of free water soluble monosaccharides from the flowers of *Linum usitatissimum*.

A brief review of uptodate literature on chemical examination of selected plants, has been described respectively in each concerned chapter.

The work represented in the thesis has been carried out in the Chemical Laboratories of Dayanand Vedic Post-graduate College, CHAI, under the Supervision of Dr. G. S. Niranjan, D.Phil.,F.I.C.S., Department of Chemistry, Dayanand Vedic (P.G.) College, CHAI.

A brief summary of the entire work has been submitted separately along with the thesis, according to the requirements of ordinances for Ph.D. degree of Kurukshetra University.

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The realisation of the object would have been an uphill task without the encouraging help from Dr. Y. S. Srivastava, Dr. S. K. Katiyar, Dr. B. C. Sikrorie, Mr. R. K. Gupta, Sri S. J. Srivastava for providing valuable suggestions during my research period and during the writing of the thesis.

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CONTENTS

CHAPTER - I

INTRODUCTION

1.1 INTRODUCTION

From the time immemorial diseases have been the cause of mark before human sufferings. Plants have been proved beneficial from the earliest times in curing various ailments and diseases. A continued search for medicinal plants during the last several centuries has given us a bulk of medicinal plants which are of great use in the treatment of diseases and promotion of health.

Medicinal properties of plants are due to the presence of definite chemical constituents in them. Medicinal plants may sometimes contain some toxic substances. That is why, the use of plants in their natural states is not proper and isolation of active principles in pure state from medicinal plants is very essential. The chemical investigation in the field of Natural Products gained pace with the advent of the use of developed new techniques like chromatography[1], spectrometry[2,3,4] and various physico-chemical methods. These modern techniques are useful to isolate active organic compounds even when they are present in a very small quantity.

There are many families and genera in the vast flora world which have high medicinal values and have not been investigated as yet for their active principles. The new researches on these plants may prove quite beneficial to cure human ailments.

Various chemical constituents obtained from the plants are classified into many groups. A brief account of the review on the

classes of compounds investigated from the plants, which have been incorporated in the present thesis is given below

I.2 - Polysaccharides.
I.3 - Sterols.
I.4 - Flavonoids.
I.5 - Free sugars.

I.2 POLYSACCHARIDES

Polysaccharides are most important component of all living organisms and highly distributed among the higher order of land plants and sea weeds. They are present in fungi exoskeleton of insect and crustaceans, in the capsules of microorganism, in cartilage, in animal joint fluids etc.

Polysaccharides are macromolecular compounds, composed of several monosaccharide units, usually linked through oxygen to give complex composition. They are hydrophillic colloids of high molecular weight, some completely soluble in water, other swell and absorb considerable amount of water without dissolving.

Gums and mucilages are complicated polysaccharide polymers and differ in the respect that the former are characterised as plants exudates while the latter are isolated from various plant organs by extraction with water.

Plant gums and mucilages have been known and in use since very early times, reference being made to them in the Bible, and they seem to have been of commercial value for several thousand

years, especially in India, Asia, Africa, Australia, and China.

There is no agreement as to the origin of gum exudates, but they play an important role in the physiology of plants, in animal and microorganism as surface material and regarded as food reservers[5] in such the same manner as starch in many plants and glycogen in animals or as agent for holding water[3,6]. The plant is believed to synthesize the gum exudates in order to seal off the infected section of the plant and prevent further invasion of the tissues[7,8,9]. Whatever the exact origin and mode of formation of the gums may be , it is reasonable to believe that gum exudates are formed by some type of enzymic polymerization and not by direct polymerization.

Gums and mucilages are used in wide range of industries like cosmetics[10,11,12] , pharmacy[13,14], textiles[15,16], adhesives[17], food products[18,19,20], paper[21,22] and in many other fields.

A polysaccharide is isolated from the plant by extraction with cold or hot water, water containing a little acetic acid and the precipitation of the soluble portion with the excess of ethanol. The polysaccharide is purified to remove the inorganic ions and proteinous impurities[23] by repeated precipitation with ethanol from acidified aqueous solution.

The homogeneity of the polysaccharide is checked by fractional precipitation[24], zone electrophoresis[25,26], and acetylation and deacetylation[27]. A mixture of polysaccharides can be separated over a cellulose column[28,29], while the acidic polysaccharides may be fractionated as their complexes[30]. Ion exchange columns[31] are

also used effectively for the fractionation but the methylated gums are separated over alumina[32]. Electrophoretic separation of polysaccharides has been achieved mainly in borate buffer[33,34], but acetate buffer[35,36] and citrate buffer[36] have also been used. With the help of membranes or filters of desired porosity[37] polysaccharides may be fractionated. The unwanted polysaccharides of the mixture may be destroyed with specific enzymes[38] followed by denaturation of enzyme with heat, alkali and alcohol. The fractionation of polysaccharides may also be achieved by gel filtration[39] and molecular sieve[40].

The purified polysaccharide is subjected to preliminary determination of lignin, ash content, methoxyl, acetyl, primary hydroxyl and carbonyl groups and they are estimated after the detection of nitrogen, sulphur, phosphorus and halogens which may be present in the polysaccharide.

The optical rotation of the polysaccharide is measured by means of usual polarimeter[41] or photoelectric spectropolarimeters[42] The configuration of glycosidic linkage in oligosaccharides can be correlated to optical rotatory power by applying Hudson's rule of isorotation[43].

The molecular weight of the polysaccharide having terminal reducing group can be determined by estimating it with C^{14} labelled sodium cyanide[44], sodium hypoiodite[45], ferricyanide[46], and periodate oxidation studies[47]. Physical methods like viscosity[48], light scattering[49], osmotic pressure[50] are also used to determined the molecular weight of the polysaccharide.

The hydrolysis of the polysaccharide with mineral acids under different conditions provides information regarding the nature of linkages present between sugar moieties. The complete acid hydrolysis of the polysaccharide results in the liberation of monosaccharides which can be separated by paper[31] or column chromatographic[32] techniques. They are identified by their R_f values, co-chromatography with authentic samples, melting points and by preparing their crystalline derivatives. Partial acidic hydrolysis with dilute mineral acids (0.01 - 0.1N) results in degradation of the polysaccharide into less complicated molecules which can easily be identified. Oligosaccharides, obtained by partial hydrolysis, can be separated by paper chromatography and their structure is determined by the usual process of methylation, followed by the hydrolysis and identification of methylated sugars, periodate oxidation and enzymic hydrolysis. Enzymic degradation[33] provides various information about the polysaccharide.

The sugars may be quantitatively estimated by microvolumetric method, spectrophotometric method or colorimetric method. Recently an extensive use of gas liquid partition chromatography[34-36] in the separation and estimation of sugars has been reported.

The polysaccharide is subjected to periodate oxidation to obtain the information regarding the nature of end groups and types of glycosidic linkage[37] present. It has been observed that the 1,2-diol groups in 1 → 2 or 1 → 4 linked and 1,2,3-triol groups in the 1 → 6 linked anhydrohexose units are oxidised by one and two moles of periodate respectively, liberating one mole of formic

acid but the units having 1 →3 linkages with no 1,2-diol system are not effected. Thus by determining the consumption of periodate and amount of formic acid liberated, various informations regarding the structure may be obtained.

The methylation studies serve the valuable information regarding the types of linkages between sugar moieties in a polysaccharide. The method consists in the methylation of the polysaccharide followed by hydrolysis to give methylated sugars. The nature and the quantitative determination of the methylated sugars provide information on the relative proportions of non-reducing end groups, the degree of branching, the type of interchain linkages and the nature of the main chain linkages in the polysaccharide. Methylation is usually carried out by means of Haworth's method[58] followed by Purdie's method[59]. The methylated product is hydrolysed in two steps, first the methanolic hydrogen chloride[60] or with 85-90% formic acid[61] and finally with the mineral acids. The methylated sugars are separated on paper and identified by their R_{TMG}[62] values, optical rotations and melting points of their crystalline derivatives. The methylated sugars are quantitatively estimated by titrating them with alkaline hypoiodite or by colorimetric method. These polysaccharides which are soluble in dimethyl sulphoxide, may be very efficiently methylated[63] in fewer steps by using methyl iodide and silver oxide.

In the present thesis, the chemical examination of two complex water soluble polysaccharides, a galactoxylan (isolated from the seeds of Zizyphus jujuba) and a galactomannan (isolated from the seeds of Phaseolus aureus) have been described in Chapter II and Chapter III respectively.

I.3 STEROLS

They are crystalline compounds, and contain an alcoholic group. The structure of the sterols are based on the 1:2 cyclopentanophenanthrene skeleton. The sterols give characteristic Liebermann-Burchard reaction.

The plants have variety of closely related sterols called phytosterols. They occur in the plants in free state or as esters of higher fatty acids or sometimes as glycosides called steroline. Many of them are isolated from the unsaponifiable portion of oils and fats. The well known phytosterols are stigmasterol, β -sitosterol and ergosterol.

The sterols are found to be physiologically important substances, play various roles in life process and have great importance in animal metabolism, hormones, co-enzymes, bile acids and provitamin-D.

The investigator has been able to isolate a β-sitosterol from defatted matters of the seeds of Daucus carota. The chemical study of this sterol has been described in Chapter IV of the thesis.

I.4 FLAVONOIDS

Flavonoids form the largest group of naturally occurring oxyheterocyclic compounds as pigments. They possess structure based upon the $C_6 - C_3 - C_6$ carbon skeleton. In which two benzene rings joined by a three carbon link which is formed into a γ-pyran ring. The various classes of flavonoid compounds - flavones,

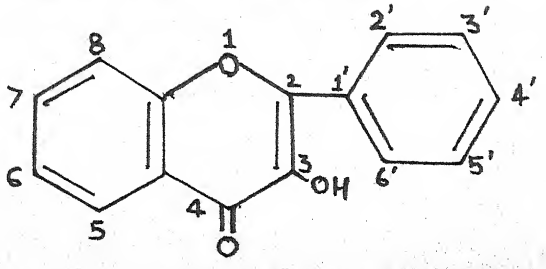
isoflavones, flavonols, flavanonones, dihydroxyflavonoles, flavanones, isoflavenones, chalkones, dihydroxychalkones, auzones, anthocyanidins and leucoanthocyanidins, differ from one another only by the state of oxidation of this 3-C-link.

Flavonoids are present in plants in the free state as well as in the form of glycosides, containing either sugars on more than one hydroxyl group or disaccharide (biosids) and trisaccharides. Most plants contain more than one glycoside of any aglycone.

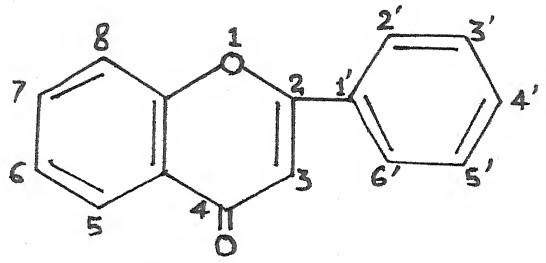
It is supposed that flavones protect plants from harmful ultraviolet radiations or from loss of important materials by autooxidation and one is tempted to believe physiological functions of the flavonoid pigments based upon their colours are related. to the role of flowers in reproduction[64]. These compounds were found to be of great medicinal importance as bacteriostatic[65] and insecticidal etc.

I.4.1 FLAVONES AND FLAVONOLS

The flavones and flavonols are naturally colouring matters. Their structure is based on that of 2-phenyl-4-chromone. The flavones and flavonols differ in the respect that latter has a hydroxy group at position -3. The basic skeleton of flavone and flavonol may be respresented as.



Flavonol skeleton



Flavone skeleton

The structure of these compounds was not properly elucidated until 1891 although Morin was isolated as early as 1814. In 1891 Hertzig[66] reported the structure of quercetin. Afterwards (1893)[67] the structure of chrysin was determined. Today nearly one hundred flavones and flavonols have been isolated, the latter class making nearly two-third of the total[68].

These compounds occur naturally in free state or as glycosides. The position occupied by a sugar unit in glycosidic linkage, plays an important part due to which a glycoside exhibits difference in properties as solubility and capacity to form complexes with metals. Unlike anthocyanins in which the sugar residue is usually present at position 3 and 5, the sugar moiety in flavones and flavonols is generally attached to a hydroxyl group at position 3 or 7.

These compounds have been found to be highly physiologically active. The flavonol glycoside rutin has been described for its therapeutic properties. The insecticidal action of polyhydroxy flavones and their ethers and the action of flavones on isolated

enzyme system[69] have been studied.

The author has been able to isolate a flavone glycoside and a flavonol compound from the seeds of Sesbania aculeata. The chemical study of these colouring substances has been described in Chapter IV of the thesis.

1.3 FREE WATER SOLUBLE SUGARS (CARBOHYDRATES)

Carbohydrates are an important class of naturally occurring substances and are found universally distributed among plants, animals, and micro-organisms. The name carbohydrate arose from the fact that the first compounds of this group to be studied were found to have an empirical formula $C_x(H_2O)_y$ and were believed to be hydrates of carbon. Since that time, however carbohydrates which do not have hydrogen and oxygen present in the proportion to form water (e.g., rhamnose, $C_6H_{12}O_5$) has been discovered, and other carbohydrates containing nitrogen and sulphur are also known. Although it is difficult to define such a heterogeneous group, the carbohydrates may be thought of as polyhydroxy aldehydes or ketones and derivatives of them.

Catabolism of carbohydrates provides the major share of the energy requirement for maintenance of life and performance of work. The metabolism of carbohydrates is of central importance to organism, individually and collectively. Basically all organic food-stuff are ultimately derived from the synthesis of carbohydrates through photosynthesis.

Carbohydrates are divided into three basic categories : Monosaccharides, oligosaccharides and polysaccharides. Monosaccharides have three to nine, usually either five or six, carbon atoms and contain only one aldehydic or ketonic functional group. The oligosaccharides are oligomers of monosaccharides linked by formation of glycosidic linkages. These generally contain two to eight or ten monomeric units. Polysaccharides are frequently molecules of great size and may have molecular weights of many million. They contain more than ten monomeric units.

In the present thesis in Chapter V the study of water soluble monosaccharides from the flowers of Linum usitatissimum is incorporated.

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CHAPTER - II

A NEW WATER SOLUBLE POLYSACCHARIDE FROM THE SEEDS OF ZIZYPHUS JUJUBA

II.1. The subject matter of this Chapter is isolation and characterisation of a neutral water soluble polysaccharide from the seeds of *Zizyphus jujuba*.

The plant *Zizyphus jujuba* Lank. commonly known as Berberi (Indian jujube). The cultivated tree is called 'Pawandi' or 'Bandiber'. This plant belongs to the family Rhamnaceae[1], a shrub or moderate-size tree, almost evergreen, usually armed. Young branches and flowers densely tomentose. Leaves variable, 1 ÷ inches long, ovate-oblong or sub-orbicular, obtuse or acute, entire or serrulate, dark green and glabrous above, clothed beneath with dense pale-coloured tomentum. Prickles solitary and straight or in pairs with one of them shorter and recurved, rarely wanting. Flowers greenish-yellow somewhat foetid, arranged in short axillary, subsessile cymes. Calyx glabrous within. Petals clawed, with an oblong hooded lamina. Disk 10-lobed. Ovary 2 celled, styles 2, connate to the middle. Drupe ½ - ¾ inches or longer, globose oblong or ovoide, orange or red when ripe. Stone 2 - celled, with a hard thick bony shell.

Indigenous and naturalized throughout India and in Ceylon, wild and cultivated, also in Tropical Africa, the Malay Archipelago, China and Australia.

Various parts of the tree are used medicinally[2]. The fruit is largely eaten by natives and it is much valued in times of scarcity and it was consider/ed to purify the blood and aid digestion. Root decoction used in fever and as a powder applied to old wounds and ulcers. Bark considered to be a remedy in diarrhoea. The

~~of~~ use of Egyptian wood in ancient civilization is reviewed, and the abilities of many these species to have resisted termite attack was studied. This specimen is 3000 - 4000 years old. The oldest Egyptian wood belongs to the genus Zizyphus[3].

II.2. The Brief Review of Chemical Examination of this plant in the Literature is Described as given below :

Genus	Plant species	Constituents	Parts	References
1. Zizyphus	-	Vitamin C Content	-	(1936)[4]
2. Zizyphus	-	Anthraquinone derivative	Wood	(1938)[5]
3. Jujube (Florida grown)	-	Carotene and ascorbic acid content	-	(1948)[6]
4. Jujube (Korea)	-	Lysine, aspartic acid glycine, asparagine, glutamic acid & galactose	-	(1969)[6]
5. Zizyphus	Talonl (Bolkat) (Balco)	Lignin and -cellulose	Wood (Philippine)	(1932)[7]
6. Zizyphus	Spina Christi	Tannin	Bark	(1938)[8]
7. Zizyphus	Rugosa	Starch moisture 68.4%, ash content 1.68%, starch prepd. 1.29%	Seeds	(1949)[9]
8. Zizyphus	Jaoserio	Detergent analysis	Bark	(1961)[10]
9. Zizyphus	Jaoserio	Jujubic acid	Bark	(1957)[11]
10. Zizyphus	Xylophora	Betulinic acid	Bark & wood	(1961)[12]
11. Zizyphus	Xylophyrus	Tannins & oleanolic acid	Fruits	(1963)[13]
12. Zizyphus	Xylophyrus	(-)-Leucoanthocyanin	Fruits	(1968)[14]
13. Zizyphus	Conoplia	Two new basic peptide Zizyphine I, Zizyphinine II, Betulinic	Root & bark	(1963)[15]

Genus	Plant species	Constituents	Parts	References
		acid, D-glucose, D-fructose, sucrose.		
14. Zizyphus	Oenoplia	Constitution of Zizyphinine	Root & Bark	(1969)[16]
15. Zizyphus	Mauritiana	Two peptide alkaloids Mauritine (A) & Mauritine (B)	-	(1972)[17]
16. Zizyphus	Fructus	Water soluble carbohydrates.36.1% fructose, 32.2% D-glucose.14.8% oligosaccharide, 1.4% arabinose, 2.3% galacturonan.	Fruits	(1969)[18]
17. Zizyphus	Nummularia	Na, K, Ca, Mg, Fe, Al, Cu, and Zn trace mineral constituents	-	(1970)[19]
18. Zizyphus	Vulgaris	Fatty acid and resin acids from ether extract	Bark	(1934)[20]
19. Zizyphus	Vulgaris	Anaesthetics	Leaves	(1941)[21]
20. Zizyphus	Vulgaris	Chinese drug (extracted oil 89.16% fatty acids of which 90.7% are unsatd.(Palmitic acid & Phytosterol) inaluding oleic, α-linoleic acids and β-linoleic acids.	Seeds	(1936)[22]
21. Zizyphus	Vulgaris	Betulinic acid ($C_{30}H_{46}O_2$)	Seeds	(1946)[23]
22. Zizyphus	Jujube	Leucocyanidin & Leucopelargonidin and Betulinic & Cosmethic acids	Bark & wood	(1961)[12]
23. Zizyphus	Jujube	Caryl alcohol, Alkaloids, Protopine & Berberin	Leaves	(1936)[24]
24. Zizyphus	Jujube	Rutin	Leaves	(1968)[25]

Genus	Plant Species	Constituents	Parts	References
25. Zizyphus	Jujube	Five alkaloids of 13 membered cyclopeptide alkaloidal ring structure	Leaves	(1973)[26]
26. Zizyphus	Jujube	Tannins, Anthraglucosides, Saponins, Alkaloids, coumarins, anthocyanins, flavone glucosides and mucilage.	Fruits & Leaves	(1968)[27]
27. Zizyphus	Jujube	Carbohydrates, Carotene, tannins, flavone glycosides, saponins, lipids, resins and mucilage	Fruits &	(1969)[28]
28. Zizyphus	Jujube	Oil, contained oleic, linoleic, arachidic and behenic acids.	Seeds	(1953)[29]
29. Zizyphus	Jujube	Essential amino acid contents	Seeds	(1969)[30]
30. Zizyphus	Jujube	Sapogenin (Abelin lactone)	Seeds	(1970)[31]
31. Zizyphus	Jujube	Saponin (Jujuboside B_1 structure elucidation by carbon-13 nuclear magnetic resonance		(1978)[32]
32. Zizyphus	Jujube	Auxin	Endosperms	(1969)[33]

A number of chemical compounds have been already reported in the above literature, but no attempt has been made for the isolation and structure elucidation of polysaccharides of Zizyphus jujuba. Because of the medicinal and industrial values of the plant, it was considered worthwhile to isolate and establish

the structure of their polysaccharide isolated from the seeds of Z. jujuba.

II.3 STRUCTURAL ELUCIDATION OF NEUTRAL WATER SOLUBLE POLYSACCHARIDE

RESULTS AND DISCUSSION

The polysaccharide was isolated from the defatted seeds of Z. jujuba, extracting with water (1% acetic acid) and precipitating with ethanol. The polysaccharide was purified by repeated precipitation with ethanol to get a white fibrous mucilage with minimum ash content (0.6%). The homogeneity of the polysaccharide was checked by.

(i) Fractional precipitation.
(ii) Zone - electrophoresis.
(iii) Acetylation and deacetylation.

The polysaccharide was dissolved in water and separated into two fractions by fractional precipitation with different volumes of ethanol. Both the samples were analysed quantitatively by the method of Hirst and Jones[45]. The results were essentially identical showing the homogeneity of the polysaccharide.

The polysaccharide was acetylated with acetic anhydride by the usual method to give the acetylated product. $[\alpha]_D^{21}$ - 30° (in acetone, C, 1.1%). Deacetylation of the product gave a polysaccharide having the same optical activity as the original one. This confirmed the homogeneity of the polysaccharide.

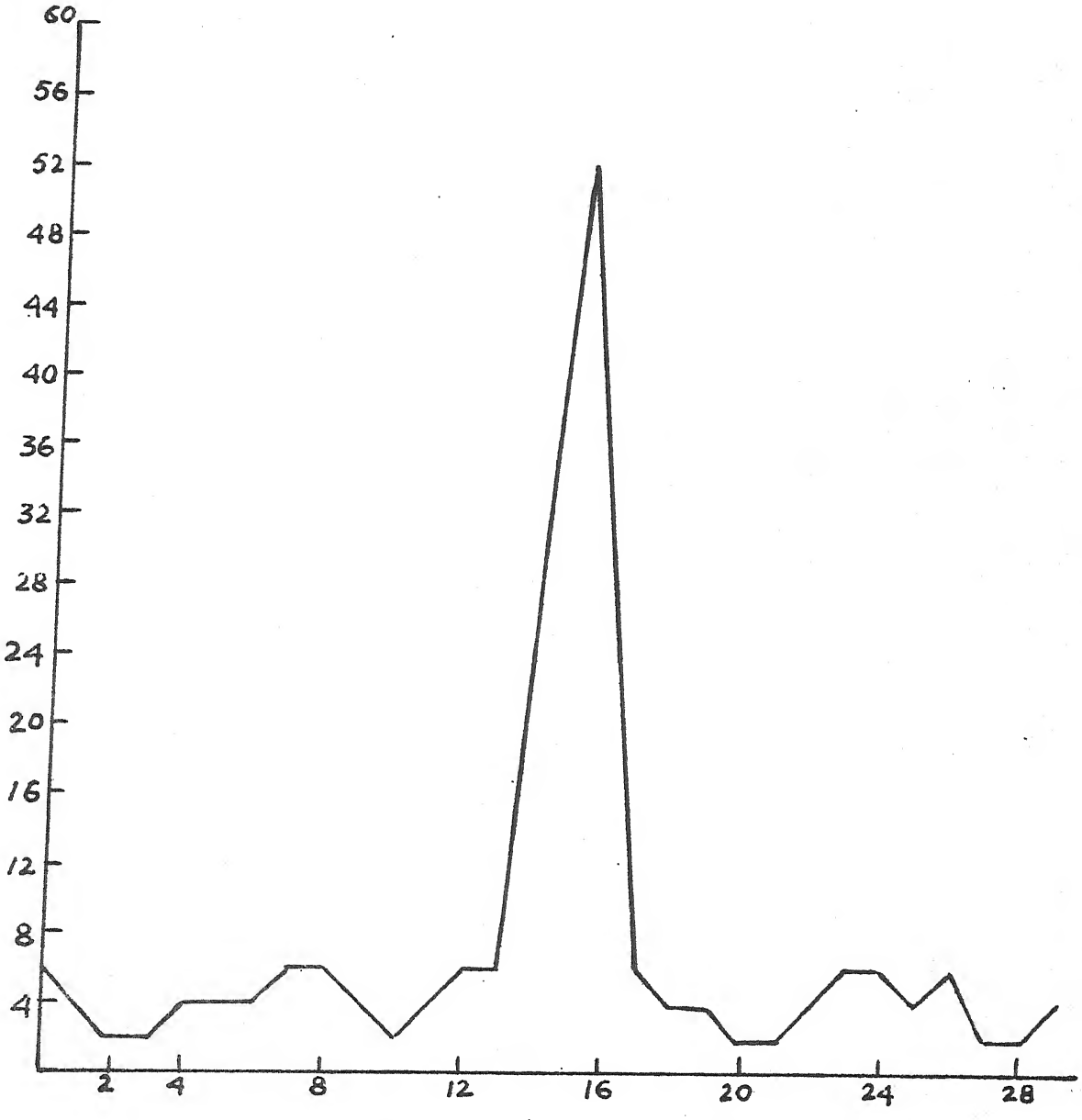

60
56
52
48
44
40
36
32
28
24
20
16
12
8
4
2
4
8
12
16
20
24
28
Segment number

Fig. (1)

Another portion of polysaccharide was separated by zone-electrophoresis in borate buffer (pH 9.3). The paper chromatogram was cut into 1.0 cm segments, which were numbered consecutively from anodic end down to cathodic end. Each segment was eluted with distilled water, treated with phenol-sulphuric acid reagent and the absorbance of characteristic orange yellow colour was measured in a Klett-Summerson photoelectric colorimeter, using filter No. 30. A plot of the absorbance against segment number showed only a single sharp peak (Fig. - 1) indicating the polysaccharide to be homogeneous.

The polysaccharide was slowly soluble in water, $[\alpha]_D^{24} = 91.2^\circ$ (in water, C, 0.8 g per 100 ml of solution), ash content 0.5%. The polysaccharide was found to be free of nitrogen, sulphur, and halogens. The methoxyl, uronide and acetyl percentage were found to be negligible.

The complete acid hydrolysis of the polysaccharide with 2% sulphuric acid followed by the paper chromatographic analysis of the hydrolysate revealed the presence of two sugars, D-galactose and D-xylose. The identity of the sugars was confirmed by their specific optical rotations, preparation of their crystalline derivatives and co-chromatography with authentic samples.

The quantitative estimation of monosaccharide components by periodate oxidation, taking ribose as a reference sugar, showed that galactose and xylose are present in the molar ratio , 1:5 in the polysaccharide. The graded hydrolysis of the polysaccharide with 0.05N sulphuric acid and subsequent paper chromatographic

analysis of the hydrolysates, taken out at various intervals, revealed that D-galactose was liberated first followed by the liberation of D-xylose. This shows that most of the xylose units are linked together forming the backbone (main chain) of the polysaccharide and galactose units are linked as terminal groups.

The polysaccharide was methylated first by Haworth method using dimethyl sulphate and alkali[37] followed by Purdie's method[38] with methyl iodide and silver oxide to give a methylated product, $[\alpha]_D^{21}$ - 36° (in chloroform, C, 1 g. per 100 ml of solution), OMe, 44.6%. The complete hydrolysis of the methylated polysaccharide and paper chromatographic analysis of the hydrolysate in Solvent A, revealed the presence of four methylated sugars. The methylated sugars were separated on a preperative scale by chromatography on Whatman No.3 filter paper. The following methylated sugars were identified.

(I) 2-O-methyl-D-xylose.

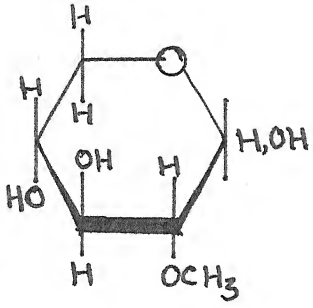
(II) 2,3-Di-O-methyl-D-xylose.

(III) 2,3,4-Tri-O-methyl-D-xylose.

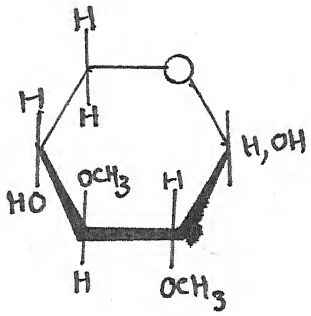
(IV) 2,3,4,6-Tetra-O-methyl-D-galactose.

Methylated sugar, I, had R_{TMG} value in solvent A, 0.39, $[\alpha]_D^{25}$ - 24° (in water, C, 2%), m.p. 130-32°. It formed 2,O-methyl-D-xylose anilide, m.p. 123-24°, $[\alpha]_D^{25}$ + 213° (in ethyl acetate, C, 0.8%). Its diacetate, 2-Omethyl, 3,4-diacetate had m.p. 73-77°, $[\alpha]_D^{25}$ - 38.5° (in chloroform, C, 2.5%). Thus the above observations confirmed that the methylated sugars, I, is 2-O-methyl-D-xylose.

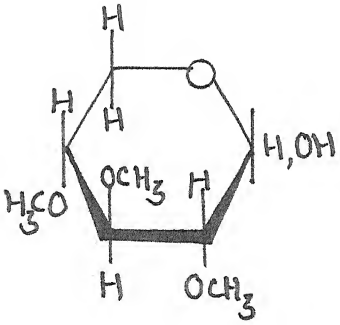
Methylated sugar, II, was obtained as a syrup, R_{TMG} in solvent A, 0.76, $[\alpha]_D^{20}$ + 22.2° (in water, C, 4.33%), OMe, 34.6%.


H
H
H
OH
H
H,OH
HO
H
OCH3

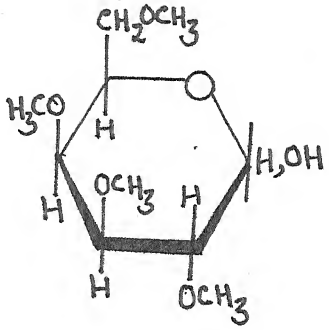
I


H
H
H
OCH3
H
H,OH
HO
H
OCH3

II


H
H
H
OCH3
H
H,OH
H3CO
H
OCH3

III


CH2OCH3
H3CO
H
OCH3
H
H,OH
H
H
OCH3

IV

It formed anilide derivative, 3,3-di-O-methyl-D-xylopyranosyl anilide, m.p. 130°, $[\alpha]_D^{21}$ + 192.3° (in ethyl acetate, C, 0.223%), which shows that the methylated sugar, II, is 2,3,-di-O-methyl-D-xylose.

Methylated sugar, III, was also a syrup, could not be re-crystallised, R_{EMG} in solvent A, 0.92, $[\alpha]_D^{19}$ + 19.2° (in water, C, 0.39). On treatment with ethanolic aniline it gave 2,3,4-tri-O-methyl-D-xylopyranosyl anilide, m.p. 94-96°, $[\alpha]_D^{22}$ - 83° (in ethanol, C, 2%). The sugar in this fraction was thus identified as 2,3,4-tri-O-methyl-D-xylose.

Methylated sugar, IV, R_{EMG} in covent A, 0.90, $[\alpha]_D^{25}$ + 124° (in water, C, 0.6%), m.p. 72-73°. On treatment with ethanolic aniline gave , 2,3,4,6-tetra-O-methyl-N-phenyl-D-galactosylamine, m.p. 189-90°, $[\alpha]_D^{25}$ - 80° (in acetone, C, 1%). Therefore, the identity of methylated sugar, IV, is established as 2,3,4,6-tetra-O-methyl-D-galactose.

The quantitative estimation of methylated sugars, by the method of Hirst and Jones[60] showed that the sugars, I, II, III, and IV were present in the molecular ratio 5:8:2:3.

The appearance of 2,3,4,6-tetra-O-methyl-D-galactose, IV and 2,3,4-tri-O-methyl D-xylose, III, on hydrolysis of methylated polysaccharide indicates that all galactose units and xylose (2 units) in the polysaccharide occupy terminal position as non-reducing end groups. A large proportion of, II, 2,3-di-O-methyl-D-xylose (8 moles) indicates that the backbone of the polysaccharide consists of D-xylose units linked through 1 → 4 linkages.

Detection of 2-O-methyl-D-xylose, I, (5 moles) shows that five xylose units in the main chain per repeating unit of the polysaccharide are linked at position 3 in addition to 1- and 4- positions.

Determination of terminal groups by periodate oxidation and subsequent titration of formic acid liberated corresponds to 0.2066 moles of formic acid per 100 g of the polysaccharide. On the basis of methylation studies, the simplest repeating unit of polysaccharide, is supposed to consist of 18 sugar moieties of which 3 units of galactose and 2 units of xylose form terminal groups, considering such a repeating unit, the terminal groups were found 28.33% as determined by periodate oxidation studies, which is in close agreement to that revealed by methylation studies (27.71%).

During the periodate oxidation studies, the oxidised polysaccharide was taken out from the reaction mixture after 72 hours and hydrolysed after destroying the periodate. The paper chromatographic examination of the hydrolysate showed that the presence of xylose was quite prominent, while no galactose could be detected. The paper chromatography of the hydrolysate of the oxidised polysaccharide taken out from the reaction mixture after 96 hours showed the absence of both the sugars. It reveals that galactose units were completely oxidised within 72 hours, whereas xylose units were oxidised only after 96 hours. The considerable difference in the rates of oxidation of the component sugars is due to steric effect resulting from the branched structure of the polysaccharide. The present knowledge , however, indicates that this phenomenon is most likely due to cyclic acetal formation[46].

The partial acid hydrolysis of the polysaccharide followed by paper chromatographic separation on preparative scale afforded six oligosaccharides which were detected as follows :

1. 3^2-β-Xylobiosyl xylobiose (1 → 4-O-β-D-xylopyranosyl-1 → 3-O-β-D-xylopyranosyl-1 → 4-O-xylopyranose) or 3^3-β-Xylosyl xylotriose (O-β-D-xylopyranosyl-(1 → 3)-O-β-D-xylopyranosyl (1 → 4)-O-β-D-xylopyranosyl-(1 → 4)-D-xylopyranose).

2. 3^2-β-Xylosylxylobiose (-O-β-D-xylopyranosyl-(1 → 3)-O-β-D-xylopyranosyl-(1 → 4)-D-xylopyranose).

3. Xylotriose (-O-β-D-xylopyranosyl-(1 → 4)-O-β-D-xylopyranosyl-(1 → 4)-O-β-D-xylopyranose).

4. Rhodymenobiose (-O-β-D-xylopyranosyl-(1 → 3)-O--D-xylopyranose).

5. Xylobiose-(-O-β-D-xylopyranosyl-(1 → 4)-O-β-D-xylopyranose.

6. O-β-D-galactopyranosyl-(1 → 4)-O- -D-xylopyranose.

Oligosaccharide (1), $[\alpha]_D^{21}$ - 57.8° (in water, C, 3%) found chromatographically pure in two solvents F and B. The complete acid hydrolysis followed by paper chromatographic analysis revealed the presence of only xylose units in the oligosaccharide. The molecular weight 360.3, of the oligosaccharide of corresponded to a tetrasaccharide of pentoses. Partial acid hydrolysis of tetrasaccharide gave oligosaccharides, 3^2-β-xylosyl xylobiose,

xylotriose (-O-β-D-xylopyranosyl-(1 → 4)-O-β-D-xylopyranosyl-(1 → 4)-O-β-D-xylopyranose), rhodymenabiose and xylobiose, corresponded, the the oligosaccharides (2), (3), (4) & (5) respectively. A (1 → 3) linkage in the oligosaccharide was also confirmed by periodate oxidation. The consumption of 3.2 moles of metaperiodate, with the liberation of 2.16 moles of formic acid per mole of the oligosaccharide. Had, all the sugar moieties in the tetrasaccharide been linked by (1 → 4) linkages the tetrasaccharide would have consumed 6 moles of periodate instead of 3.2 moles. The hydrolysis with the enzyme emulsin and the negative rotation indicated that the xylose units in the oligosaccharide were linked through β - linkages. On the basis of these experimental evidences, the oligosaccharides have been identified as (1 → 4)-O-β-D-xylopyranosyl-(1 → 3)-O-β-D-xylopyranosyl-(1 → 4)-D-xylopyranose, i.e. 3^2-β-xylobiosylxylobiose or O-β-D-xylopyranosyl-(1 → 3)-D-xylopyranosyl- (1 → 4)-O-β-D-xylopyranosyl-(1 → 4)-D-xylopyranose, i.e. 3^3-β-xylosylxylotriose. Fig. - 2(a) and 2(b).

Oligosaccharide (2), m.p. 222°, $[\alpha]_D^{21}$ - 31° (in water, C, 2.98), was chromatographically pure in solvents F and B. The molecular weight 420 corresponded to a trisaccharide of pentoses. Acid hydrolysis of the oligosaccharide yielded only xylose. The anomeric configuration of non-reducing xylose units were found to be 'β' by enzymic hydrolysis and negative rotation. Partial acid hydrolysis yielded, xylobiose, rhodymenabiose, corresponding to oligosaccharides (5) and (4) respectively and xylose which were identified by co-chromatography with the authentic samples. Periodate oxidation studies revealed the consumption of 4.3 moles of metaperiodate with the liberation of 2.1 moles of formic acid.

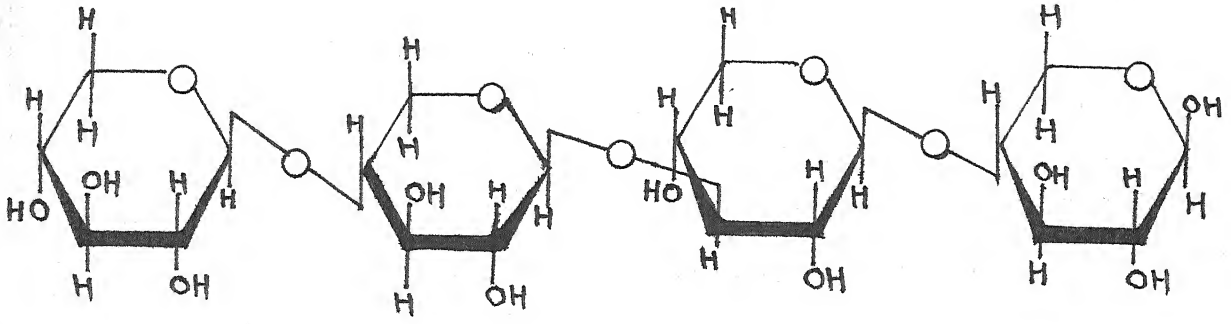


Fig. - 2(a).

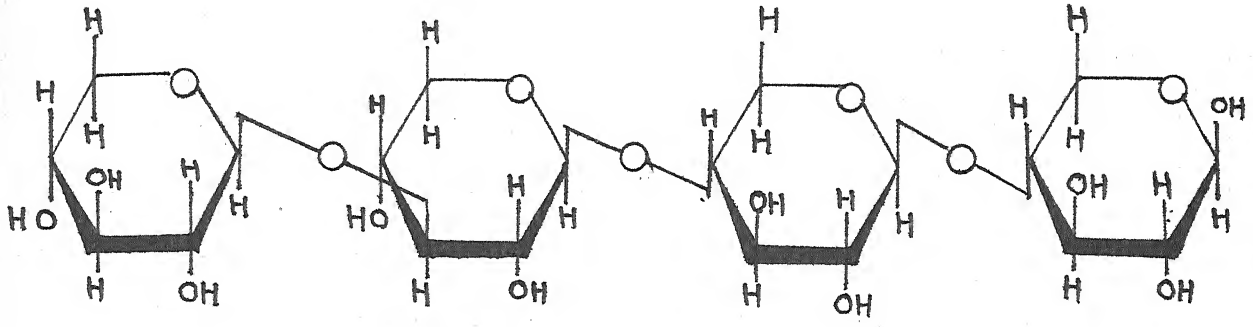


Fig. - 2(b).

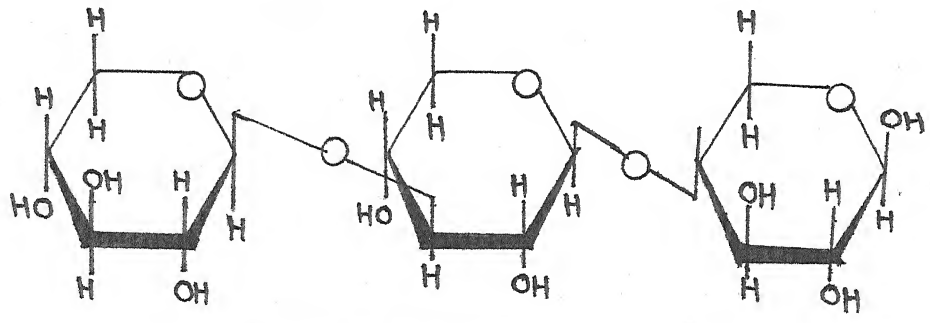


Fig. - 3.

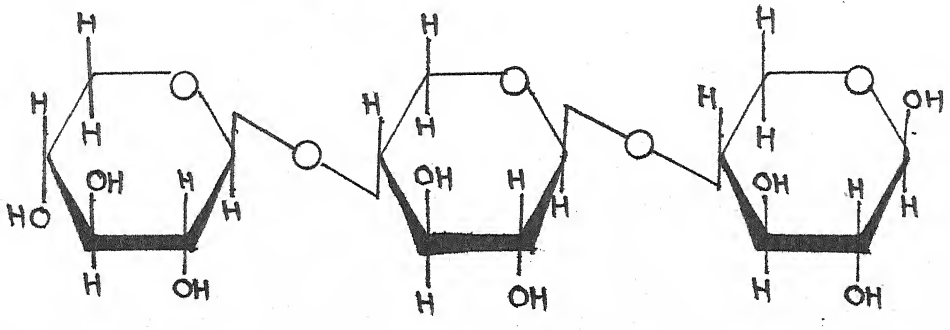


Fig. - 4.

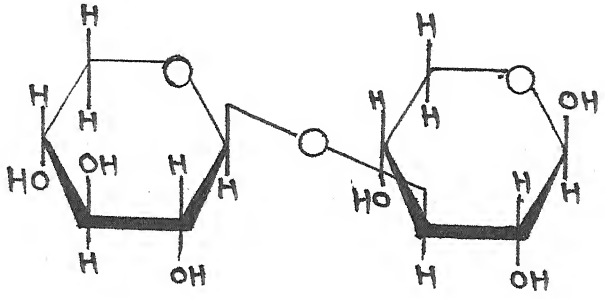


Fig. - 5.

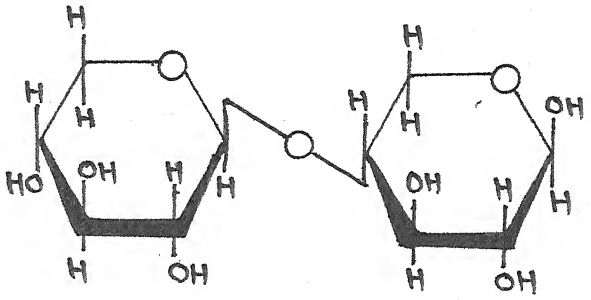


Fig. - 6.

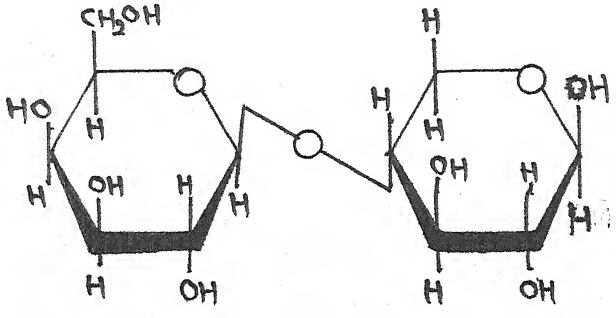


Fig. - 7.

Hence the oligosaccharide was identified to be O-β-D-xylopyranosyl-(1 → 3)-O-β-D-xylopyranosyl-(1 → 4)-D-xylopyranose i.e. 3^2-β-xylosylxylobiose. (Fig. - 3).

Oligosaccharide (3) , a crystalline form having the physical constants identical with those reported for (1 → 4)-O-β-D-xylopyranosyl-(1 → 4)-O-xylopyranose, m.p. 203-05°, $[\alpha]_D^{22}$ - 46° (in water, C, 1.0%). It was found to be chromatographically pure in solvent F and B. Acid hydrolysis of the oligosaccharide yielded only xylose and partial acid hydrolysis gave xylose and xylobiose. The identity of these oligosaccharides was confirmed by co-chromatography with their authentic samples. The molecular weight was found to be 423, which corresponded to trisaccharide of pentose units. Enzymic hydrolysis with emulsin and negative rotation showed that the xylose units were linked through β-linkages. The periodate oxidation studies afforded the liberation of 2.2 moles of formic acid and consumption of 3.21 moles of periodate per mole of the trisaccharide. On the basis of above evidences , the oligosaccharide was identified to be O-β-D-xylopyranosyl-(1 → 4)-O-β-D-xylopyranosyl-(1 → 4)-O-β-D-xylopyranose. (Fig. - 4).

Oligosaccharide (4) a crystalline sugar, m.p. 190°, $[\alpha]_D^{22}$ - 20.4° (in water, C, 2.9%), was found to be chromatographically pure in two solvent systems F and B. The sugar on acid hydrolysis yielded only xylose while the molecular weight of the sugar 296 corresponded to a pentose disaccharide. Enzymic hydrolysis with emulsin showed the presence of β-linkage between the two xylose units. The periodate oxidation showed the consumption of 3.24 moles of metaperiodate with liberation of 1.18 moles of formic acid per mole of the sugar. The oligosaccharide is,

therefore identified to be O-β-D-xylopyranosyl-(1 → 3)-O-β-D-xylopyranose. (Fig. - 5). The identity was confirmed by co-chromatography with an authentic sample.

Oligosaccharide (5), m.p. 186-87°, $[\alpha]_D^{30}$ - 25° (in water, C, 3.3%), was chromatographically pure in solvent F and B. Acid hydrolysis showed the presence of xylose only. The molecular weight of the sugar was 298, corresponded to a disaccharide of xylose units. The periodate oxidation showed the liberation of 2.21 moles of formic acid with the consumption of 4.31 moles of metaperiodate per mole of oligosaccharide. Hence the oligosaccharide was assigned the structure O-β-D-xylopyranosyl-(1 → 4)-xylopyranose. (Figg- 6). The identity was further confirmed by co-chromatography with an authentic sample.

Oligosaccharide (6), $[\alpha]_D^{30}$ + 14° (in water), m.p. 190-91°, was shown to be chromatographically pure in solvent I. On acid hydrolysis revealed the presence of galactose and xylose. The quantitative estimation by the method of Hirst and Jones[45] showed the molar ratio to be 1:1 between the two sugars in the oligosaccharide. The molecular weight 296, showed it, to be a disaccharide. Periodate oxidation studies afforded liberation of 2.12 moles of formic acid and consumption of 4.14 moles of periodate per mole of the oligosaccharide. (Fig - 7).

On the basis of the results obtained so far particularly from methylation studies, graded and partial acid hydrolysis, the following valuable information could be derived :

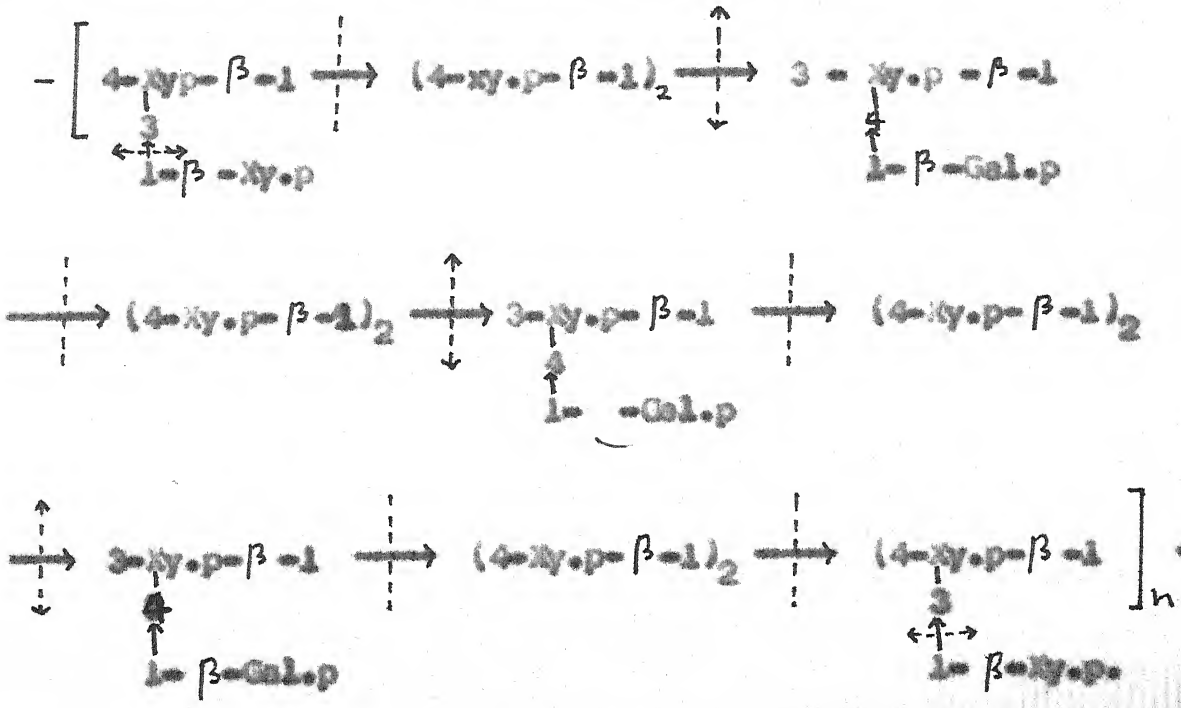
(i) The main chain of the polysaccharide consists of β-(1 → 4) and β-(1 → 3) linked xylose units.

(ii) All the galactose units are present as terminal groups and linked in the main chain through β-(1 → 4) linkages.

(iii) Two xylose units per repeating unit of the polysaccharide are also linked as a side chain and linkages between main chain xylose units and side chain xylose units are β-(1 → 3).

(iv) From the above information, it is also clear that the galactose units in the side chain are linked at the same xylose units in the main chain which linked through β-(1 → 3) linkages in the main chain.

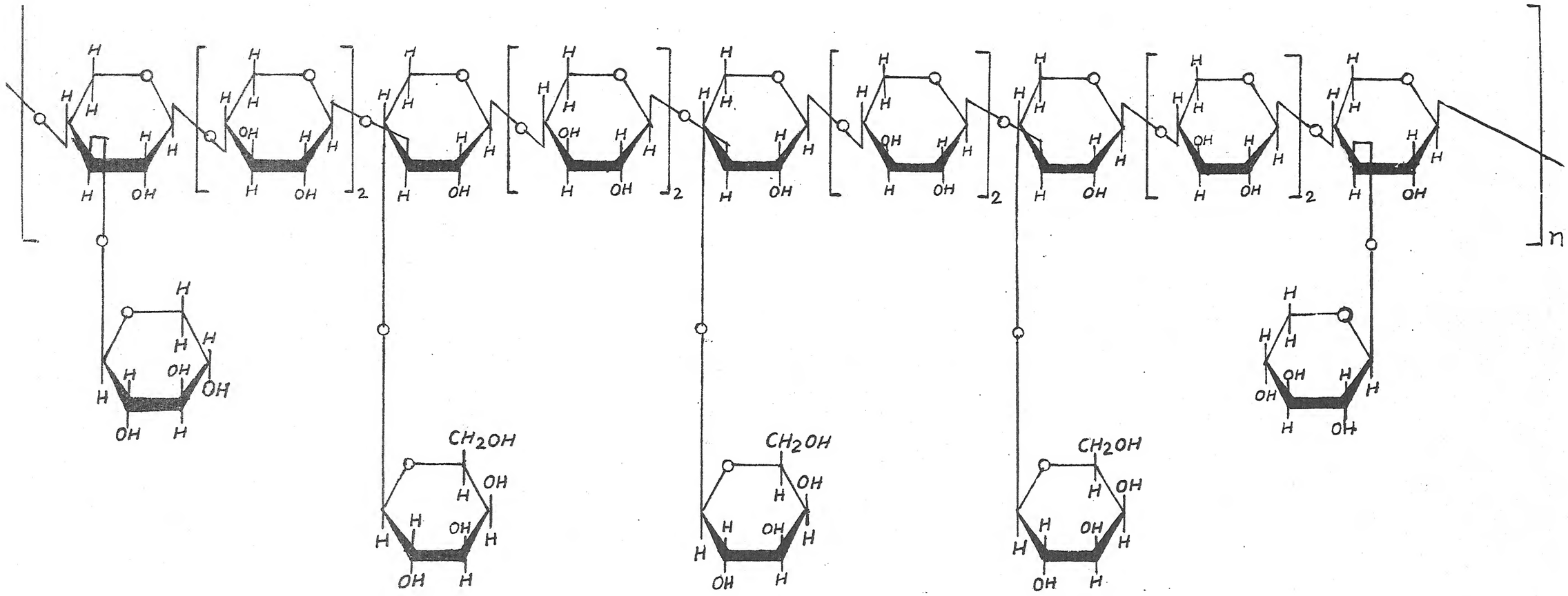
Taking all the experimental evidences into consideration together with the structures of different oligosaccharides, the following most probable structure has been assigned to the polysaccharide from the seeds of Zizyphus jujuba.



The above structure contains 18 units of monosaccharides per repeating unit which fully explains the formation of oligosaccharides as obtained by partial acid hydrolysis and agrees well with the analytical data of the polysaccharide. The dotted and doubly arrowed dotted lines show the probable mode of fission of the linkages during the partial acid hydrolysis. The arrowed dotted lines indicated secondary hydrolysis.

The polysaccharide such as described above should consume 18 moles of metaperiodate with the liberation of 3 moles formic acid per repeating unit of 18 sugar units. The actual consumption of periodate 18.21 and liberation of formic acid 3.04 moles have been determined for per repeating unit of the polysaccharide, which are in close agreement to the calculated values.

Similar other structures may be possible but they are less probable because the formation of oligosaccharides as obtained in the present case might not be possible.



STRUCTURE OF POLYSACCHARIDE FROM THE SEEDS OF ZIZYPHUS JUJUBA

II.2 EXPERIMENTAL

All evaporation were carried out under reduced pressure at low temperature unless specified otherwise. Residues were dried in vacuum at room temperature over anhydrous calcium chloride. All specific rotations are equilibrium values and all melting points are uncorrected. Paper chromatography was performed at room temperature by descending technique on Whatman No.1 filter paper unless stated otherwise, using following solvent system :

(A)	n-Butanol - ethanol - water	(4:1:5)[34]
(B)	n-Butanol - acetic acid - water	(4:1:5)[35]
(C)	n-Butanonol- iso-propanol - water	(11:6:3)[36]
(D)	Benzene - ethanol - water	(160:47:15)[37]
(E)	Butanone - water	(11:1)[38]
(F)	Ethyl acetate - pyridine - water	(11:4:3)[39]
(G)	Ethyl acetate - pyridine - water	(2:1:2)[40]
(H)	n-Butanol - ethanol - water	(40:10:19)[41]
(I)	n-Butanol - ethanol - water	(5:1:4)[42]

The spots were located by spraying a chromatogram with aniline hydrogen phthalate[43] and heating it at 110-20° for 10-15 minutes. Spectrophotometric determination were carried out by a modification of phenol - sulphuric acid method[44]. Klett-Summerson photoelectric colorimeter was used for measureing the absorbance.

II.3 ISOLATION OF THE POLYSACCHARIDE

The dried and crushed seeds (1.0 kg) were extracted successively with petroleum ether (60-80°) and ethanol. The extracted seeds were dried and then suspended in distilled water (1 litre) containing 1% acetic acid. The mixture was stirred mechanically for 8-10 hours to extract the mucilage as much as possible and squeezed out through a muslin cloth. The process was repeated six times when practically no precipitate was obtained by adding the extract to an excess of ethanol. The combined extracts were filtered thrice

through a thick cotton pad, placed over a cloth in a Buchner funnel to remove the suspended fine particles. The clear mucilage solution so obtained was added slowly to a large excess of ethanol with constant vigorous stirring when a fibrous colourless precipitate of the crude polysaccharide was obtained. It was filtered, washed with ethanol, followed by absolute ethanol and dried in vacuum at room temperature (38 g ; ash 3.13%).

II.6 PURIFICATION

The dried crude polysaccharide was dissolved in distilled water (2 litres) containing 1% acetic acid with constant stirring The solution was filtered and was added very slowly to ethanol (8 litres) with constant and vigorous stirring and kept overnight. The precipitated polysaccharide was filtered and the above process was repeated four time, to get a white fibrous mucilage. (25 g ; ash 0.5%).

II.7 HOMOGENEITY OF THE POLYSACCHARIDE

The homogeneity of the polysaccharide was checked by the following methods.

II.7.1 (a) Fractional Precipitation

The pure mucilage (4 g) was dissolved in distilled water (500 ml). It was then added slowly to ethanol (500 ml) and the precipitated polysaccharide (Fraction I) was filtered, washed with ethanol followed by absolute ethanol and dried in vacuum. The filtrate was treated with another 1000 ml of ethanol with stirring and precipitated polysaccharide (Fraction II) was filtered,washed and dried in vacuum. Both the fractions along with the original polysaccharide were hydrolysed seperately with 2% sulphuric acid. The sugar present in each hydrolysate were first identified by

paper chromatography with authentic sugars using solvent (c) and then separated on two sheets of Whatman No.1 filter paper using the same solvent. The sugars were eluted with water and estimated quantitatively by periodate oxidation method[45]. The sugars eluted from one sheet were estimated by titration of formic acid liberated with standard alkali solution whereas the sugars from the other sheet were estimated by the method of consumption of periodate. The ratio of D-galactose and D-xylose in both fractions was found almost the same (1:5), indicating the purified polysaccharide to be homogeneous.

II.7.2 (b) Acetylation and Deacetylation

The pure polysaccharide (1.5 g) was mixed thoroughly with anhydrous sodium acetate (10 g) and mixture was suspended in acetic anhydride (30 ml). After refluxing over a water-bath for 18 hours, the mixture was cooled to room temperature, and poured over crushed ice with constant stirring and then left overnight. The greyish-white precipate was filtered, washed with water and dried in vacuum. The dried mass was then dissolved in minimum quantity of acetone and the solution was poured slowly in distilled water, where upon a fine fibrous precipate was obtained. This precipitate was filtered, washed and dried in vacuum 1.12 g , $[\alpha]_D^{21} = 50^\circ$ (in acetone, C, 1.1%).

The dried acetylated polysaccharide (0.9 g) was dissolved in acetone (32 ml) and 30% potassium hydroxide solution (32 ml) was added to it. The deacetylation was carried out in the usual manner[46] by refluxing the mixture over a water-bath for six hours. The viscous solution was poured slowly with stirring into 3% ethanolic acetic acid (300 ml) to precipitate the polysaccharide. The precipitate was filtered and was again precipitated by dissolv-

ing in water and dried. 0.28 g. $[\alpha]_D^{21}$ - 90° (in water, C. 1%).

The original polysaccharide $[\alpha]_D^{21}$ - 91.2° (in water, C. 0.8%) and the polysaccharide obtained after deacetylation had almost the identical specific rotations indicating the homogeneity of the polysaccharide.

II.7.3 (c) Zone - Electrophoresis

A strip support (15 cms x 45 cms) of Whatman No.1 filter paper was marked with a pencil in middle to indicate the starting line. 0.3% solution of polysaccharide (50 ml) was placed on starting line as a compact band. After drying at room temperature the strip was sprayed with borate buffer (pH 9.3) and suspended h horizontally in the electrophoresis tank containing two electrode compartments each having approximately 400 ml of borate buffer (pH 9.3). After electrophoresis at 260 V and 12.5 mA for 6.5 hours, the paper strip was dried. It was then cut lengthwise into 1 cm segments, which were numbered to the cathode end. The material from each numbered strip was eluted with water (6 ml) and filtered through glass wool. The filtrate (5 ml) was placed in a hard glass boiling tube with 8.5% aqueous phenol (1 ml). To the tube, concentrated sulphuric acid (15 ml) was added rapidly. The tubes were allowed to cool at room temperature. The absorbance of characteristic yellow orange colour was measured in a Klett-Summerson photoelectric colorimeter using filter No.50. A blank was also run under the same conditions but without polysaccharide.

The reading so obtained were plotted against the segment number counted from the anode end to the cathode end. Only one sharp peak was obtained indicating the polysaccharide to be homogeneous.

TABLE - 1

Segment No.	Klett reading of eluate	Blank Klett reading	Corrected Klett reading	Absorbance
1	25	22	3.0	0.006
2	23	22	1.0	0.002
3	23	22	1.0	0.002
4	23	21	2.0	0.004
5	23	21	2.0	0.004
6	23	21	2.0	0.004
7	25	22	3.0	0.006
8	24	21	3.0	0.006
9	23	21	2.0	0.004
10	23	22	1.0	0.002
11	25	23	2.0	0.004
12	24	21	3.0	0.006
13	25	22	3.0	0.006
14	40	23	17.0	0.034
15	48	22	26.0	0.052
16	39	23	16.0	0.032
17	25	22	3.0	0.006
18	23	21	2.0	0.004
19	24	22	2.0	0.004
20	24	23	1.0	0.002
21	23	22	1.0	0.002
22	23	21	2.0	0.004
23	25	22	3.0	0.006
24	24	21	3.0	0.006
25	25	23	2.0	0.004
26	25	22	3.0	0.006
27	22	21	1.0	0.002
28	22	21	1.0	0.002
29	25	23	2.0	0.004
30	24	22	2.0	0.004

Absorbance was measured on 5 ml portion of coloured solution.

$$\text{Absorbance} = \frac{2 \times \text{Klett reading}}{1000}.$$

II.8 ASH CONTENT

The dried polysaccharide (0.2 g) was ignited in a silica crucible previously heated to a constant weight. After ignition the crucible was cooled in a desiccator and weighed. From the weight of residue (0.0010 g), the ash content was calculated to be 0.5%.

II.9 PHYSICAL AND CHEMICAL EXAMINATION

It was a fibrous white powdered, very light in weight, slowly soluble in water. $[\alpha]_D^{21} = 91.2^0$ (in water, C, 0.8 g per 100 ml of solution). For the purpose of optical rotation, the solution was filtered through a sintered funnel to get a clear solution and the amount of polysaccharide in the solution was determined colorimetrically. The polysaccharide was found to be free of nitrogen, sulphur and halogens. It did not reduce Fehling's solution.

II.10 EXAMINATION OF FREE SUGARS

The polysaccharide was examined for free sugars by applying three spots of its solution in water on a strip of Whatman No.1 filter paper (15 cm x 45 cms). The paper was developed in solvent (A) for 36 hours, dried and cut lengthwise into three strips, each containing one spot. The three strips were sprayed with three different reagents using naphthoresorcinol and trichloroacetic acid (gives colour with ketoses only)[47] on one, aniline hydrogen phthalate[48], on the second and silver nitrate in acetone followed by ethanolic sodium hydroxide[49], on the third. The first two paper dried in

the oven at 120° and the third was air-dried. None of the strip showed any spot, hence the polysaccharide did not contain any free sugar.

II.11 METHOXYL GROUP DETERMINATION

The percentage of methoxyl groups was determined by the method of Belcher, Fildes and Nutten[49] and was found to be 0.74%.

II.12 ACETYL GROUPS DETERMINATION

The method by Belcher and Godbert[50] was followed for the determination of acetyl group percentage with and without mucilage. Found acetyl 0.90%.

II.13 URONIDE CONTENTS DETERMINATION

The uronide contents were found to be negligible by the semi-micro method of Barker, Foster, Siddiqui and Stacey[51].

II.14 HYDROLYSIS OF POLYSACCHARIDE AND DETERMINATION OF MONOSACCHARIDES

The purified mucilage (1.2 g) was dissolved in 2N sulphuric acid (100 ml) and was hydrolysed on a water-bath for about 24 hours. The hydrolysate was neutralised with barium carbonate, filtered and concentrated under reduced pressure. The hydrolysate was examined for monosaccharide as described on next page.

II.14.1 (a) Paper Chromatography

The spots of the hydrolysate were applied on two sheets of Whatman No.1 filter paper. The papers were developed separately in solvents (A) and (B) by descending unidimensional technique. The chromatograms were air-dried and sprayed with aniline hydrogen phthalate. On heating them in an oven at 120°, each chromatogram showed two spots. The R_f and R_G values of the two spots corresponded to D-galactose and D-xylose as given in the following Table.

TABLE - 2

Sugar identified	Solvent (A) R_G found	R_G given 1.52	Solvent (B) R_f found	R_f given 35
D-Galactose	0.08	0.07	0.16	0.16
D-xylose	0.14	0.15	0.27	0.28

G = 2,3,4,6-Tetra-O-methyl-D-glucose.

The identity of two sugars was further confirmed by co-chromatography with authentic samples of the sugars.

II.14.2 (b) Column Chromatography

A portion of hydrolysate was dissolved in a small amount of aqueous methanol (1:1) and adsorbed over a well washed column of cellulose (1" x 15"). The column was left over-night and the separation was effected with solvent (A) and several fractions (15 ml) each were collected. Each fraction was analysed by paper chromatography with authentic samples of D-galactose and D-xylose

in solvent (B). The fraction 1 - 10 containing same sugar were combined together and concentrated to give D-xylose. It was re-crystallised from aqueous methanol, $[\alpha]_D^{30}$ + 17.5° (in water ; C, 1.14%). The melting point of the sugar was found to be 143-44° The following derivative was prepared.

D-Xylose Phenyl Osazone Derivative

The osazone of the sugar was prepared by heating (250 mg) of sugar , 50 mg of phenyl hydrazine hydrochloride and 0.3 g of sodium acetate dissolved in 5 ml of water in a test tube and heated for 30 minutes on a boiling water-bath. Precipitate of the osazone started appearing after 7 minutes. The flocculent precipiate was separated with water, recrystallised from 50% ethanol, m.p. 160-61° resembling to an authentic.

The fraction 15 - 30 were mixed and concentrated to give D-galactose. It was recrystallised from aqueous methanol, $[\alpha]_D^{25}$ + 79.2° (in water, C, 0.5%). The melting point of the sugar were found to be 167°. The following derivatives were prepared.

(i) D-Galactose Phenyl Hydrazone

Found	Given (Lit.)[53]
m.p. 153-54°	154-55°

(ii) N-p-Nitrophenyl-D-Galactosylamine

In a microtest-tube were taken galactose (25 mg), p-nitro-aniline (25 mg), a drop of glacial acetic acid and 2 drops of metha-nol : water (8:1 v/v). The mixture was boiled for 8 minutes and kept overnight in a refrigerator. The crystalline product was filtered, washed with cold ethanol, ether and dried in vacuum. It

melted at 218-19° after recrystallisation from methanol. Lit.[54] m.p. 219°.

II.14.3 (c) Thin-layer Chromatography

The plates were prepared from slurry of silica gel G in 0.1N solution of boric acid and the spots of hydrolysate along with benzene:acetic acid:methanol (1:1:3)[55] and air-dried. These plates were sprayed with aniline hydrogen phthalate reagent. On heating them at 120° in an oven, two spots corresponding to D-galactose and D-xylose were observed.

II.15. QUANTITATIVE ESTIMATION OF MONOSACCHARIDE

The method due to Hirst and Jones[45] was applied for quantitative estimation of component sugars of the polysaccharide.

The polysaccharide (200 mg) was dissolved in 2N sulphuric acid (20 ml) in a 250ml round bottom flask. The flask was then heated for 24 hours on a water-bath. After cooling to room temperature the hydrolysate was diluted to 30 ml and then D-ribose (20 mg) was added to it. The whole solution was shaken well and transferred to a beaker. The flask was washed well with water, and the washings were transferred to the beaker. The solution was netralised with barium carbonate and filtered. The filtrate and the washing of barium carbonate were concentrated and then made upto 10 ml.

Six sheets (30 x 45 cms) of Whatman No.1 filter paper were used as paper chromatograms. Three guide strips (4 x 45 cms) two on either edges and one in centre, were marked on each paper. A portion of above solution was placed along the starting line

(8 cms away from the upper edge) of the three sheets, whereas the remaining three sheets were used as blanks. A guide spot was placed in the centre of each guide strip. All the sheets were developed in solvent (C) for 48 hours. After drying the chromatograms, guide strips were cut lengthwise, sprayed with aniline hydrogen phthalate and heated in an oven at 120° to locate the position of sugars. With the help of these guide strips, appropriate sections of unsprayed portion were cut along with the blank strips of same dimensions from the blank chromatograms. Each section (with and without sugar) was cut into small pieces and extracted separately with 10 ml of hot water. The eluted sugars were then oxidised with 0.25 M sodium metaperiodate (5 ml). The liberated formic acid was titrated with standard alkali, after destroying the excess of metaperiodate with ethylene glycol (2 ml), using methyl red as indicator. Blank readings were substracted to get the titre values.

TABLE - 3

Sugar	Value of alkali* used (in ml)			Corresponding amount of sugar (in mg)		
	A	B	C	A	B	C
Galactose	3.00	4.29	3.92	0.886	1.253	1.158
Xylose	14.62	20.40	18.88	4.501	6.280	5.812
Ribose	1.76	2.44	2.26	0.542	0.751	0.696

* Strength of sodium hydroxide = $\frac{N}{121.5}$.

Assuming complete recovery of D-ribose, the above results indicate that in the polysaccharide D-galactose and D-xylose are in the molar ratio of 1:3.

II.16 GRADED HYDROLYSIS[36] OF THE POLYSACCHARIDE

The polysaccharide (100 mg) was dissolved well in 0.05N sulphuric acid (20 ml) and the hydrolysis was carried out over a boiling water-bath. The hydrolysates, taken out at various intervals, were examined chromatographically without removal of sulphuric acid using solvent (3) for the purpose of irrigation of the paper. Results are given in Table - 4.

TABLE - 4

Time (in minutes)	Sugar identified	No. of other spots
5	Galactose (Faint)	
10	Galactose + xylose (Faint)	
15	Same as above	
20	Same as above	
25	Same as above	
30	Same as above	Two spots of oligo-saccharide.
60	Same as above	Two spots of oligo-saccharide.
90	Same as above	Three spots of oligo-saccharide
120	Galactose + xylose	Four spots of oligo-saccharide.
180	Same as above	Same as above
240	Same as above	Same as above
420	Same as above	Same as above

During graded hydrolysis of the polysaccharide galactose was found to be liberated first followed by xylose. The ~~result~~ earliest release of D-galactose and simultaneously of D-xylose

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During graded hydrolysis of the polysaccharide galactose was found to be liberated first followed by xylose. The ~~result~~ earliest release of D-galactose and simultaneously of D-xylose

(faint) leads to the conclusion that D-galactose are present as terminal groups and some units of D-xylose are also present as terminal groups instead of main chain of the polysaccharide. As galactose is liberated earlier than xylose, this is most probably attached to the main chain by more easily hydrolysable linkages.

II.17 METHYLATION OF POLYSACCHARIDE

The polysaccharide was methylated first by the method of Parikh, Ingle and Bhide[37] followed by Purdie's method[38].

The polysaccharide (8.0 g) dissolved in minimum amount of water and then taken in a conical flask fitted with B-24 joint. Dimethyl sulphate (40 ml) and 40% sodium hydroxide (80 ml) were added dropwise with constant stirring with magnetic stirrer. The temperature was maintained between 40-50°. After repetition of the above procedure, the solution was concentrated under reduced pressure and filtered to remove the sodium sulphate. The filtrate was again concentrated to a thick syrup and dissolved in acetone. This was then methylated by repeating the above procedure thrice. The finally concentrated solution was extracted thoroughly with chloroform. The extracts were dried over anhydrous sodium sulphate and the solvent distilled off under reduced pressure. The partly methylated product was brownish mass. (6.92 g). $-OCH_3$, 35.5%. $[\alpha]_D^{21}$ = 40° (in chloroform, C, 1.2 per 100 ml of solution) .

The partly methylated polysaccharide was further methylated by Purdie's method. The partly methylated polysaccharide (6.5 g) was dissolved in methanol (36 ml) in a conical flask fitted with three necked multiple adapter. The temperature was maintained at

40-50° by placing the conical flask, fitted with air-condenser having fused $CaCl_2$ -tubes in a trough containing water over the magnetic stirrer. Methyl iodide (9 g) and silver oxide (6 g) were added with continuous stirring in several equal instalments, each after half an hour interval. After the final addition the reaction mixture was heated for four hours on a water-bath under reflux and then filtered after cooling the contents. The silver salts were exhaustively extracted with chloroform under reflux. The combined filtrate and extracts were evaporated under reduced pressure and the resulting syrup was remethylated thrice under the same conditions. The fully methylated polysaccharide was obtained as a deep brown coloured product . (3.1 g) $-OCH_3$, 44.6% . $[\alpha]_D^{21}$ = 36° (in chloroform, C, 1.0 g per 100 ml of solution) .

II.18 HYDROLYSIS OF THE METHYLATED POLYSACCHARIDE AND IDENTIFICATION OF METHYLATED SUGARS

The hydrolysis of methylated polysaccharide was carried by slight modification of method due to Bouveng et al[59]. The methylated polysaccharide (100 mg) was dissolved in 85% formic acid (20 ml) and solution was refluxed for 4 hours on a water-bath. The solution was then cooled and concentrated under reduced pressure and traces of formic acid were removed under vacuum. It was dissolved in 0.25 M sulphuric acid (10 ml) and the hydrolysis was carried out for 16 hours on a water-bath. The hydrolysate was cooled , neutralised with barium carbonate and filtered. The residue was washed with water followed by ethanol. The combined solutions were concentrated under reduced pressure to light brown syrup. The methylated sugars were separated on Whatman No.1 filter

paper using solvent (A). The chromatograms showed four spots, after spraying with aniline hydrogen phthalate and drying at 120°. The R_{TMG} (TMG = 2,3,4,6-tetra-O-methyl-D-glucose) value of each methylated sugar was calculated in solvent (A) and R_g value was calculated in solvent (I). These values were compared with that given in literature as shown in the following table.

TABLE - 5

Methylated sugars identified	Solvent (A)	
	R_{TMG} found	R_{TMG} given[34,61]
2-O-Methyl-D-xylose	0.39	0.38
2,3-Di-O-methyl-D-xylose	0.76	0.74
2,3,4-Tri-O-methyl-D-xylose	0.92	0.94
2,3,4,6-Tetra-O-methyl-D-galactose	0.90	0.88

II.19 QUANTITATIVE ESTIMATION OF METHYLATED SUGARS

The methylated polysaccharide (300 mg) was hydrolysed as described above. After hydrolysis, glucose (60 mg) was added to hydrolysate. It was then neutralised with barium carbonate and filtered. The residue was washed with ethanol. The filtrate and washings were concentrated under reduced pressure to a syrup. A portion of the syrup was dissolved in acetone and applied on three sheets (A, B, and C) of whatman No.1 filter paper. Each having three guide strips. The papers were irrigated with solvent (D) along with three blank sheets. After development of chromatograms and locating the sugars on guide strips, appropriate sections, containing sugars were cut from the unsprayed portion of the chromatograms. The sugars were eluted with 20 ml of water.

The methylated sugars were estimated by alkaline hypoiodite method[60]. The eluted portions were taken in 50 ml conical flasks separately provided with ground glass joint stoppers and a solution (2 ml) containing 0.2% sodium bicarbonate and 0.2% sodium carbonate was added Solution of iodine (0.1N, 2 ml) was then added to the reaction mixture and the flask was stoppered. The experiments as corresponding blank eluates were also carried out in the same way. After three hours, the reaction mixture was acidified cautiously with 2 N sulphuric acid and 15% potassium iodide solution (2 ml) was then added to it. The liberated iodine was titrated against 0.01N sodium thiosulphate solution using starch as indicator. The results are given in Table - 6.

TABLE - 6

Fraction & Sugar		Volume of 0.01N hypo used (in ml)			Corresponding amount of sugar (in mg)		
		A	B	C	A	B	C
A	2,-O-methyl-D-xylose	2.36	3.18	2.74	1.722	2.321	2.000
B	2,3-Di-O-methyl-D-xylose	3.46	4.68	4.00	2.768	3.744	3.200
C	2,3,4-Tri-O-methyl D-xylose	0.78	1.06	0.92	0.678	0.922	0.800
D	2,3,4,6-Tetra-O-methyl-D-xylose	0.96	1.28	1.10	1.046	1.395	1.199
E	Glucose	1.38	1.86	1.62	1.242	1.674	1.458

The above results correspond to an average molar ratio between A : B : C : D as 2.5 : 4 : 1 : 1.5 or 5 : 8 : 2 : 3. The methylated sugars were calculated as the methyl ethers of anhydro-hexose and anhydro-pentose units i.e. $C_6H_{10}O_4$, $C_7H_{12}O_4$ and $C_8H_{14}O_4$ for mono- , di- and tri-O-methyl-D-xylose respectively and

$C_{10}H_{18}O_5$ for tetra-O-methyl-D-galactose. An average recovery of the methylated polysaccharide was found to be 99.63% assuming 100% recovery of D-glucose.

II.20 CHARACTERISATION OF METHYLATED SUGARS

The methylated polysaccharide was hydrolysed according to the method of Gaegg and Lindberg[62]. Methylated polysaccharide (4.0 g) was dissolved in 72% sulphuric acid (30 ml). The solution was kept for one hour at room temperature (25°) and then diluted to 200 ml. Further hydrolysis was carried out by heating for 4 hours on a water-bath. The solution was cooled neutralised with barium carbonate and filtered. The residue was washed with water followed by ethanol. The solutions were concentrated to a syrup under reduced pressure.

The mixture, containing different methylated sugars, was resolved into five fractions on Whatman No.3 filter paper using solvent (D). Strips, containing different individual methylated sugars, were eluted with water. The eluates were concentrated separately under reduced pressure and marked as fractions, I, II, III, and V.

II.20.1 Fraction I

Solid, R_{DMG} in solvent (A), 0.39, OMe, 18.96%, calculated for mono methyl pentose, $C_6H_{12}O_5OMe$, 18.90%, m.p. 130-32°. $[\alpha]_D^{25}$ - 24° (in water, C, 2%). Lit.[63] m.p. 135-37° $[\alpha]_D$ - 23 → 35° (in water). Lit.[64] m.p. 132-33°. $[\alpha]_D$ - 24 → 36° (in water). It formed 2-O-methyl-D-xylose anilide on treatment with ethanolic

aniline, m.p., 123-24°, $[\alpha]_D^{25}$ + 213° (in ethyl acetate solution, C, 0.8%). Lit.[63], m.p. 123-25°, $[\alpha]_D$ + 214° (in ethyl acetate).

On acetylation of sugar with anhydrous sodium acetate and acetic anhydride a greyish white precipitate was obtained. The dried mass dissolved in minimum quantity of acetone and the solution was poured slowly in distilled water, where upon a white crystalline compound 2-O-methyl-D-xylose ; 3,4-diacetate, m.p. 75-77°, $[\alpha]_D^{25}$ - 38.5° (in chloroform, C, 2.5%). Lit.[64] m.p. 78-79°, $[\alpha]_D$ - 38° (in chloroform).

II.20.2 Fraction II

Syrup, R_{TMG} in solvent (A), 0.76, found OMe, 34.8% dimethyl xylose, $C_7H_{14}O_5$, requires -OMe, 34.8%. The optical rotation of the sugar $[\alpha]_D^{20}$ + 22.2° (in water, C, 4.33%). Lit.[67], $[\alpha]_D^{15}$ + 23°.

The anilide of the sugar prepared by the method of Hampton[66] The dry syrup (200 mg) were refluxed for six hours with 1.5 ml of freshly distilled dry aniline dissolved in 10 ml of absolute ethanol. The ethanol was distilled off and the bulk of the aniline was removed under high vacuum, (5-6 mm of mercury) at 65-70° (bath temperature). The syrup mass was kept in the refrigerator for 72 hours, when tiny [illegible] crystals (plates) were observed. The adhering aniline was removed by the addition of dry ether, and the crude crystals (light brown) were filtered out, washed with ether and dried. (yield 40 mg), m.p. 138°. Lit.[67], m.p., for 2,3-di-O-methyl-D-xylopyranosyl anilide is 145° and optical

rotation $[\alpha]_D^{21}$ + 192.3° (in ethyl acetate, C, 0.222%). Lit.[67] $[\alpha]_D^{14}$ + 190° (in ethyl acetate) and in Lit.[69] $[\alpha]_D$ + 185° (in ethyl acetate).

The methoxyl content of the 2,3-di-O-methyl-D-xylopyranose anilide (recrystallised was found to be 23.2% calculated for $C_{13}H_{19}O_4N$, OMe, 24.8%). The sugar present in this fraction was identified as 2,3-di-O-methyl-D-xylose.

II.20.3 Fraction III

Syrup, it could not be recrystallised. The R_{EAG} in solvent (A) 0.92, optical rotation of sugar was found to be $[\alpha]_D^{18}$ + 18.2° (in water, C, 0.39%), Lit.[67a] is $[\alpha]_D^{15}$ + 20.3°, OMe found , 39.12%, calculated for $C_8H_{16}O_5$ is 35.35%.

The anilide of the sugar was prepared by refluxing the dry syrup (38 mg) with freshly distilled dry aniline (120 mg) for three hours in a water-bath (85-95°) in absolute ethanolic solution (5 ml). Ethanol was distilled off and the whole thing was kept in the refrigerator for seven days. The 2,3,4-tri-O-methyl-D-xylopyranosyl anilide failed to crystallise. It came out as a white ~~pre~~ powder by the addition of 3-4 drops of drys acetone. The precipitate was filtered out and dried, (yield 12 mg). The m.p. of powder was found to be 94-96°, $[\alpha]_D^{22}$ - 83° (in ethanol, C, 2%). Lit.[67] m.p. 120° , $[\alpha]_D$ - 84 ⟶ + 47° and Lit.[70] m.p. 91°. The methoxyl value of the derived anilide was found to be 33.9% ($C_{14}H_{21}O_4N$ requires, OMe, 34.8%).

The sugar in this fraction was thus identified as 2,3,4-tri-O-methyl-D-xylose.

II.20.4 Fraction IV

A solid, R_{TMG} in solvent (A), 0.90, found OMe, 51.6%. Calculated for tetramethyl hexose, OMe, 52.4%. $[\alpha]_D^{25}$ + 126° (in water, C, 0.6%). Lit.[71,72,73] for 2,3,4,6-tetra-O-methyl-galactose, $[\alpha]_D^{16}$ + 142° ⟶ + 117° (equil.) in water, C, 1.1%), m.p. 70-72°. It gave a red colour with p-anisidine hydrochloride spray in butanol and a brownish red colour with aniline hydrogen phthalate. Its treatment with alcoholic aniline gave 2,3,4,6-tetra-O-methyl-N-phenyl D-galactosylamine, m.p. 188-90°, $[\alpha]_D^{25}$ - 80° (in acetone, C, 1.0%), Lit.[71] m.p. 193-94°, Lit.[74] m.p. 192°, $[\alpha]_D$ - 77°.

II.21 PERIODATE OXIDATION OF THE POLYSACCHARIDE

II.21.1 (a) Liberation of formic acid[75] and estimation of end group

The polysaccharide (500 mg) was dissolved in water (5 ml.) and in this solution, potassium chloride (0.5 g) and 0.25M sodium metaperiodate (60 ml) were added. The volume was made upto 140 ml with water. In a blank experiment, potassium chloride (0.5 g) and (0.25M) sodium metaperiodate (60 ml) were diluted to 140 ml with distilled water. The oxidation was carried out in dark at room temperature. 5 ml. of aliquots were drawn at various intervals along with blank and excess of metaperiodate was reduced with 2 ml of ethylene glycol. The liberated formic acid was titrated against N/110 sodium hydroxide using methyl red as indicator. Results are given in Table - 7.

The data show that 0.2066 mole of formic acid was liberated (72 hours) per 100 g of polysaccharide. The amount of formic acid liberated (72 hours) corresponds to 28.33% of anhydrohexose units present as end groups. The titre value of alkali at 48, 60, and 72 hours indicated that one mole of formic acid was liberated per 531.0 g, 491.1 g and 484.03 g of the polysaccharide respectively.

TABLE - 7

Time (in hours)	Reading with blanks (in ml)	Volume of alkali used (in ml)	Corresponding amount of formic acid liberated (in mg)	Total formic acid liberated (in mg)
8	0.0	2.86	1.196	33.488
16	0.0	3.06	1.279	35.812
24	0.0	3.26	1.363	38.164
36	0.0	3.48	1.450	40.600
48	0.0	3.70	1.547	43.316
60	0.0	4.00	1.673	46.844
72	0.0	4.06	1.697	47.516
84	0.0	4.06	1.697	47.516
96	0.0	4.06	1.697	47.516

After 72 hours, 25 ml portion of reaction mixture was taken out, acidified with 2N sulphuric acid (5 ml) and then 10% potassium iodide (4 ml) was added to it. The liberated iodine was titrated immediately against N/ sodium thiosulphate solution without using starch as indicator till the solution became colourless. The solution was concentrated to 10 ml to which 2N sulphuric acid (10 ml) was added and the hydrolysis was carried out for 16 hours on a water-bath. The hydrolysate was neutralised with

barium carbonate, filtered and the filtrate was concentrated to a syrup under reduced pressure. The syrup was examined by paper chromatography using different solvents the chromatogram revealed the presence of xylose only, galactose found to be absent completely.

II.21.2 (b) Consumption of Metaperiodate[76]

The polysaccharide (250 mg) was dissolved in water (70 ml) to which 0.25 M sodium metaperiodate (40 ml) was added and the total volume was made upto 120 ml with water. A blank was also prepared with 0.25 M sodium metaperiodate (40 ml) diluted to 120 ml with water. The periodate oxidation was carried out at room temperature. 2.0 ml aliquots were withdrawn from the reaction mixture and blank at various intervals and to them 20% potassium iodide solution (2 ml) was added followed by addition of 0.5N sulphuric acid (3 ml). The liberated iodine was titrated immediately against 0.0404N sodium thiosulphate solution using starch as indicator. The reading with the polysaccharide were substracted from the corresponding readings of control experiment to get the titre values. The results are given in Table 8.

TABLE - 8

Time (in hours	Volume of hypo used (in ml).	Corresponding amount of periodate consumed (in mg)	Total periodate consumed
8	1.02	4.409	264.36
16	1.10	4.755	295.30
24	1.18	5.100	306.00
36	1.26	5.446	326.80
48	1.34	5.792	347.35
60	1.40	6.082	363.11

TABLE - 8 (Continued)

Time (in hours)	Volume of hypo used (in ml)	Corresponding amount of periodate consumed (in mg)	Total periodate consumed
72	1.46	6.301	378.07
84	1.54	6.657	399.42
96	1.54	6.657	399.42

The amount of metaperiodate consumed (84 hours) corresponds to the consumption of 0.7466 moles periodate per 100 g of polysaccharide. After 84 hours periodate oxidised solution (10 ml) was hydrolysed with 2N sulphuric acid (page 37). The hydrolysate was examined chromatographically for the presence of D-galactose and D-xylose. The chromatogram showed the absence of both the sugars.

II.22 PARTIAL ACID HYDROLYSIS OF POLYSACCHARIDE

The polysaccharide (6 g) was suspended in water (300 ml) in a three necked flask and was dissolved stirring mechanically. The hydrolysis was carried for four hours at 80° by adding 0.2N hydrochloric acid (5 ml) and the solution was stirred throughout the process. The contents, after cooling down at room temperature were poured in ethanol (2 litres) to precipitate the degraded polysaccharide. The precipitate was filtered and washed well with ethanol. The filtrate and washings were neutralised with silver carbonate with stirring. The precipate was filtered, washed with water and the combined solutions were concentrated under reduced pressure to a syrup.

II.22.1 Examination of the Precipitate

The precipitate was hydrolysed with 2N sulphuric acid for 18 hours, over a water-bath. The hydrolysate was cooled, neutralised with barium carbonate and filtered. The filtrate and washings were concentrated and examined chromatographically over Whatman No.1 filter paper using solvents (A) and (G). The chromatograms showed two spots corresponding to R_g values of D-galactose and D-xylose, which was confirmed by co-chromatography with their authentic samples. Due to small amount of precipitate, further studies were not possible.

II.22.2 Examination of the Hydrolysate

The hydrolysate was examined paper chromatographically using solvents (A), (B), (C) and (G). The chromatograms showed seven spots on spraying with aniline hydrogen phthalate and drying at 120°, indicating the presence of seven sugars.

II.22.3 Separation of Oligosaccharides

The syrup was dissolved in minimum quantity of water and applied on twenty sheets of Whatman No.3 paper as long thin band, three inches below the upper end and one inch away from the outer edges. Each paper has three guide strips, two on outer edges and one in centre. After developing the paper on solvent (a), for sixty hours, they were dried. The guide strips were cut from the chromatograms, sprayed with aniline hydrogen phthalate and dried at 120° with the help of the guide strips appropriate sections were cut from the unsprayed portion of the chromatograms and sugars

were eluted with water. In all, seven fractions were obtained.

III.22.4 Examination of Fraction I and Identification of Xylotetrose (3^2-β-Xylobiosylxylobiose or 3^3-β-Xylosylxylobiose)

R_x values were 0.62 and 0.06 in solvents (F) and (B) respectively, and xylotetrose values were 2.3 and 2.2 in solvent F and (B) respectively (Page 31). , $[\alpha]_D^{21} = 37.80^0$ (in water, C, 3%).

The sugar was hydrolysed with 2N sulphuric acid, neutralised with barium carbonate and filtered. The filtrate was concentrated and examined by paper chromatography using solvents (A) and (B). The chromatograms showed only one spot corresponding to R_x value of D-xylose. Thus sugar consist of only xylose units. Molecular weight of the sugar was determined by hypoiodite method[60], and was found to be 580.3, which corresponded to a tetrasaccharide of pentoses. Calculated molecular weigh for $C_{20}H_{34}O_{17}$, 546.

Partial acid hydrolysis of tetrasaccharide gave two trisaccharides and two disaccharides which were identified by their R_x values and co-chromatography with their authentic samples. These fractions were trisaccharides of O-β-D-xylopyranosyl-(1 → 4)-O-β-D-xylopyranosyl-(1 → 4)-O-β-D-xylopyranoside and O-β-D-xylopyranosyl-(1 → 3)-O-β-D-xylopyranosyl-(1 → 4)-D-xylopyranose, i.e. 3^2-β-xylosylxylobiose, and disaccharides of O-β-D-xylopyranosyl-(1 → 4)-O-β-D-xylopyranose, and O-β-D-xylopyranosyl-(1 → 3)-O-β-D-xylopyranose.

The presence of one (1 → 3) linkage between two xylose u units in the tetrasaccharide was further confirmed by periodate oxidation which showed the consumption of 5.2 moles of metaperiodate.

~~Iodate~~ with the liberation of 2.16 moles of formic acid per mole of oligosaccharides. The oligosaccharide was completely hydrolysed with emulsin, suggesting β-glycosidic linkages in the oligosaccharide molecule.

All the above results indicates that the oligosaccharide is O- -D-xylopyranosyl-(1 → 4)-O-β-D-xylopyranosyl-(1 → 3)-O-β-D-xylopyranosyl-(1 → 4)-O-β-D-Xylopyranose i.e. 3^2-β-xylobiosyl-xylobiose. (Fig. - 2a) or O-β-D-xylopyranosyl-(1 → 3)-O-xylopyranosyl-(1 → 4)-O-β-D-xylopyranosyl-(1 → 4)-Dxylopyranose, i.e. 3^3-β-xylosylxylobiose. (Fig. - 2b)

The identification of sugar is well supported by its constants found and reported in literature shown in the following Table - 9.

TABLE - 9

Constants	Found	Reported	References
Syrup	+	+	(77)
optical rotation	$[\alpha]_D^{21}$ - 57.8°	$[\alpha]_D^{21}$ - 56.7°±1°	(77)
R_x in solvent (F)	0.62	0.63	(39,77)
R_x Xylotetraose in solvent (S)	2.2	2.1	(35,77)

II.22.3 Examination of Fraction II and Identification of $3^2\beta$-Xylosylxylobiose

This fraction was crystallised from ethanol, m.p. 222° and $[\alpha]_D^{21}$ - 51° in water, C, 2.9%). Xylotriose values were 1.38

and 1.41 in solvents (F) and (B). R_x value in solvents (F) and (B) were found 0.70 and 0.22 respectively.

The complete acid hydrolysis with 2N sulphuric acid, subsequent neutralisation with barium carbonate and examination by paper chromatography indicated the presence of xylose only, which was further confirmed by co-chromatography with an authentic sample The molecular weight of the sugar was found to be 420 by hypoiodite method[60] which corresponded to trisaccharide of pentose units. molecular weight calculated for $C_{15}H_{26}O_{13}$. 414.

Partial hydrolysis of trisaccharide with 0.2N hydrochloric acid 100° for 30 minutes gave xylose, xylobiose, and xylotriose. Periodate oxidation studies revealed that one mole of the oligosaccharide consumed 4.3 moles of metaperiodate and 2.1 moles of formic acid liberated. It also confirmed the presence of 1 → 3 linkage between two xylose units in the oligosaccharide molecule.

The sugar was completely hydrolysed with emulsin, suggesting the presence of β-linkage. From the above observations, the sugar was identified to be O-β-D-xylopyranosyl-(1 → 3)-O-β-D-xylopyranosyl-(1 → 4)-D-xylopyranose i.e. 3^2-β-xylosylxylobiose. The constants of sugar are given below in Table - 10.

TABLE - 10

Constants	Found	Reported	References
m.p.	222°	225°	(77)
Optical rotation	$[\alpha]_D^{21}$ - 91°	$[\alpha]_D^{22}$ - 92° → -47± 1°	(77)
$R_{xylobiose}$ in solvents (F) & (B)	1.38 & 1.41	1.36 & 1.43	(77)
R_x values in solvents (F) & (B)	0.70 & 0.22	0.72 & 0.20	(77)

II.22.6 Examination of Fraction III and Identification of Xylotriose

R_x values were 0.36 and 0.10 in solvents (F) and (S) respectively. The sugar was recrystallised from 90% ethanol, m.p. 203-05°. $[\alpha]_D^{22}$ = 46° (in water, C, 1.08%).

Acid hydrolysis with 2N sulphuric acid followed by neutralisation with barium carbonate, and paper chromatographic examination showed the presence of xylose units only. The molecular weight was found to be 423 by hypoiodite method[60] which corresponded to a triesaccharide of pentose units, molecular weight calculated for $C_{15}H_{26}O_{13}$. 414. Periodate oxidation of sugar revealed the consumption of 3.21 moles of sodium metaperiodate, liberating 2.2 moles of oligosaccharide.

Partial acid hydrolysis with 0.5N hydrochloric acid 100° for 15 minutes resulted in formation of xylose and xylobiose, which were identified by co-chromatography with an authentic sample.

The sugar was completely hydrolysed with emulsin suggesting the presence of β-linkages of oligosaccharide. On the basis of above results the sugar was identified to be xylotriose, O-β-D-xylopyranosyl-(1 → 4)-O-β-D-xylopyranosyl-(1 → 4)-O-β-D-xylopyranose. The constants of sugar are given in Table - II.

TABLE - 11

Constants	Found	Reported	References
m.p.	203-05°	203-06°	(77)
Optical rotation	$[\alpha]_D^{22}$ + 46°	$[\alpha]_D^{20}$ + 45°	(78)
R_x in solvents			
(F)	0.56	0.55	(39,77)
and (B)	0.10	0.09	(39,77)

11.22.7 Examination of Fraction IV , and Identification of Rhodymenaboise

$R_{xylobiose}$ values were 1.99 and 1.03 in solvents (B) and (F) respectively, recrystallised from methanol, m.p. 196°. $[\alpha]_D^{22}$ + 39.4° (in water, C, 2.98%).

Acid hydrolysis of the sugar with 2% sulphuric acid and neutralisation of the hydrolysate with barium carbonate followed by paper chromatographic analysis in solvent (C), reveals the presence of xylose only. The molecular weight was determined by hypoiodite method[60], 296, molecular weight calculated for xylobiose, $C_{10}H_{18}O_9$, 282.

The periodate oxidation studies showed the consumption of 3.24 moles of metaperiodate with liberation of 1.18 moles of formic acid. The sugar was completely hydrolysed with emulsin, showing the presence of β-linkage. Its identity was further confirmed by preparing its phenylosazone derivative, m.p. 196-98°. $[\alpha]_D^{22}$ + 49° (in pyridine, C, 2%). And calculated for $C_{22}H_{28}O_7N_4$.

N.12.18, found 12.30%.

Constants of sugar were compared with those reported in literature as shown in Table - 12.

TABLE - 12

Sugar or derivative	Constants	Found	Reported	References
Rhodymenabiose	m.p.	190°	192-93°	(79)
-do.-	$R_{xylobiose}$ in solvent (B)	1.99	1.97	(80)
-do.-	optical rotation	$[\alpha]_D^{22}$ - 20.4°	$[\alpha]_D^{22}$ -18.4±0.6°	(80)
3-O-β-D-xylopyranosyl-D-xylose-phenyl osazone	m.p.	196-98°	194-96°	(79)
-do.-	Optical rotation	$[\alpha]_D^{22}$ + 49°	$[\alpha]_D$ + 47°	(79)

II.22.8 Examination of Fraction IV and Identification of Xylobiose

The fraction was recrystallised from aqueous ethanol, m.p. 183-84° , $[\alpha]_D^{20}$ - 25° (in water, C, 3.5%). R_x values were in solvents (B) and (F) 0.32 and 0.85 respectively.

Hydrolysis of the sugar with 2N sulphuric acid and neutralisation of the hydrolysate with barium carbonate followed by ppaer chromatography in solvent (C), revealed the presence of xylose only which was further confirmed by co-chromatography with

authentic sample. The molecular weight of the sugar was 298, calculated for $C_{10}H_{18}O_9$, 282.

The periodate oxidation of sugar consumed 4.31 moles of metaperiodate liberation with 2.21 moles of formic acid indicating the (1 → 4) linkage between xylose unit. The polysaccharide completely hydrolysed with emulsin indicating the β-linkage between two units.

Thus the oligosaccharide is a disaccharide composed of D-xylose linked through β-glycoside bond. The sugar was identified 4-O-β-D-xylopyranosyl-D-xylose, which was confirmed by preparing the phenyl osazone derivative, m.p. 204° and $[\alpha]_D^{25}$ - 51.8° (in pyridine:ethanol).

The constants of sugar are given in Table - 13.

TABLE - 13

Sugar or derivative	Constants	Found	Reported	Ref.
Xylobiose	m.p.	183-85°	185°, 187°	(81) & (73)
-do.-	Optical rotation	$[\alpha]_D^{20}$ - 25°	$[\alpha]_D^{20}$ - 25°	(81)
			$[\alpha]_D^{20}$ - 32° → - 25.5°	(82,87)
-do.-	R_x in solvent (B)	0.32	0.33	(81)
phenyl osazone	m.p.	204°	206°	(81)
-do.-	Optical rotation	$[\alpha]_D^{25}$ - 51.8°	$[\alpha]_D$ - 30°	(81)

II.22.9 Examination of Fraction VI and Identification of O-β-D-Galactopyranosyl-(1 → 4)-O-β-D-xylopyranose[65,66]

Syrup, having optical rotation, $[\alpha]_D^{30}$ + 14° (in water).

Acid hydrolysis with 2% sulphuric acid and neutralisation of the hydrolysate with barium carbonate, followed by paper chromatography, revealed the presence of D-galactose and D-xylose. The quantitative estimation by the method of Hirst and Jones[45] showed the molar ratio to be 1:1 between the two sugars in the oligosaccharide.

Periodate oxidation studies showed the consumption of 4.35 moles of periodate and liberated 2.1 moles of formic acid.

Methylation of the disaccharide followed by acid hydrolysis of the fully methylated derivative afforded 2,3,4,6-tetra-O-methyl-D-galactose and 3,4-di-O-methyl-D-xylose in equal proportions. The polysaccharide was completely hydrolysed with emulsin indicating the β-linkage between the two units.

These results ;proved that oligosaccharide was 2-O-D-galactopyranosyl-D-xylopyranose.

II.22.10 Examination of Fraction VII and Identification of D-xylose

The R_X value in ~~were in~~ solvent (B), 0.28 and R_G value in solvent (A), 0.13, m.p. 143-44°, $[\alpha]_D^{30}$ + 17.5° (in water, C, 1.14%). The sugar was identified to be D-xylose by co-chromatography with an authentic sample.

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CHAPTER - III

A NEW WATER SOLUBLE POLYSACCHARIDE FROM THE SEEDS OF PHASEOLUS MUNGO.

III.1. The present Chapter describes the isolation and structural elucidation of a water soluble neutral polysaccharide from the seeds of *Phaseolus mungo* belongs to the family Leguminosae[1].

The plant *Phaseolus mungo*, Linn. is commonly known as Urd. Stems longer and trailing, whole plant hairly with reddish-brown pubescence, which gives the foliage a lighter tint; leaves large; the pods are nearly erect, veery hairy and seeds are larger and longer than those of mung and usually dark brown and sometimes of a dull greenish.

Urd is cultivated in the Upper Gangetic Plain, especially in the Meerut and Rohilkhand Division and some part of Bundelkhand Region.

Seeds used as diet in fever and to strengthen the eye[2].

The work done in the past years on this genus was surveyed and the details of it are given in the tabular form on the next page.

Genus	Plant species	Constituents	Part	References
1. Phaseolus	acconitrifolius	Crystalline globulin	Seeds	(1937)[3]
2. Phaseolus	angularis	Kaempferol-robinobio-7-rhamnoside	Leaves	4
3. Phaseolus	trilobatus	Kaempferol-robinobio-7-rhamnoside	Leaves	5
4. Phaseolus	coccineous	Starch, amylose 2,7-amylopectin	Roots	(1963)[8]
5. Phaseolus	aureus	Uridine di-phosphate-N-acetyl glucosamine and uridine di-phosphate glucuronic acid	Seeds	(1957)[6]
6. Phaseolus	aureus	Five Oligosaccharides		(1963)[7]
7. Phaseolus	lunatus	HCN producing compounds	Seeds	8, 9, 10 (1915)[11] (1921)[12] 13, 14, 15, (1922)[16]
8. Phaseolus	lunatus	The proteins and characterisation of protein.	Seeds	
9. Phaseolus	lunatus	Enzymes	Seeds	(1928)[17]
10. Phaseolus	multiflorus	Chemical investigations on enzyme, oil, polysterol etc.	Roots	18
11. Phaseolus	multiflorus	Lipid content	Leaves	(1963)[19]

(Continued)

	Genus	Plant species	Constituents	Part	Reference
12.	Phaseolus	multiflorus	Acyl hydrolase enzyme	Leaves	(1979)[20]
13.	Phaseolus	multiflorus	Two gibberellin like compounds	Leaves	(1968)[21]
14.	Phaseolus	radiatus	Phosphorylase & Q-enzyme	Leaves	(1952)[22]
15.	Phaseolus	radiatus	Phosphoglucomutase	Seeds	(1954)[23]
16.	Phaseolus	radiatus	Phosphoglyceric acid and 2-phosphoglyceonic acid		(1954)[24]
17.	Phaseolus	radiatus	Essential amino acids; leucine, isoleucine, valine, histidine, lysine & tryptophan		(1956)[25]
18.	Phaseolus	radiatus	Moisture, ash fiber, N, etc.	Seeds	(1959)[26]
19.	Phaseolus	radiatus	Alkaline β-glycerophosphatase	Seeds	(1960)[27]
20.	Phaseolus	radiatus	Glucose, galactose, fructose, raffinose, stachyose & verbascose.	Seeds and outer seed coat	(1961)[28]

	Genus	Plant species	Constituents	Part	Reference
21.	Phaseolus	radiatus	α-Globulin & β-globulin		(1979)[29]
22.	Phaseolus	vulgaris	2-Phosphoglycolate phosphohydrolase	Leaves	(1979)[30]
23.	Phaseolus	vulgaris	Stachyose	Seeds	31
24.	Phaseolus	vulgaris	L-(+)-pibecolic acid	Seeds	(1954)[32]
25.	Phaseolus	vulgaris	Malonic acid	Seeds	(1960)[33]
26.	Phaseolus	vulgaris	Phaselic acid	Seeds	(1960)[34]
27.	Phaseolus	vulgaris	Vecilin like, leguimin like protein	Seeds	(1979)[35]
28.	Phaseolus	vulgaris	β-Fructo-furanosidase		(1964)[36]
29.	Phaseolus	vulgaris	Amino acids		(1966)[37]
30.	Phaseolus	vulgaris	2-C-methyl erythronic acid pentothonic acid & gluconic acid		(1979)[38]
31.	Phaseolus	vulgaris	Acid phosphate iso-enzyme		(1979)[39]
32.	Phaseolus	vulgaris	Six anthra-quinones	Seed coats	(1966)[40]
33.	Phaseolus	vulgrais	Sterolic compds.	Coty-ledons	(1963)[41]
34.	Phaseolus	vulgaris	Carbohydrates	-do.-	(1931)[42]
35.	Phaseolus	vulgaris	Oils	Cotyledons	(1932)[43]

Different parts of this genus have been investigated for different plant products as has already been described in literature, but no neutral polysaccharide has been mentioned uptill now. Therefore an attempt has been made for isolation and structure elucidation of the polysaccharide from the seeds of this important plant, Phaseolus mungo.

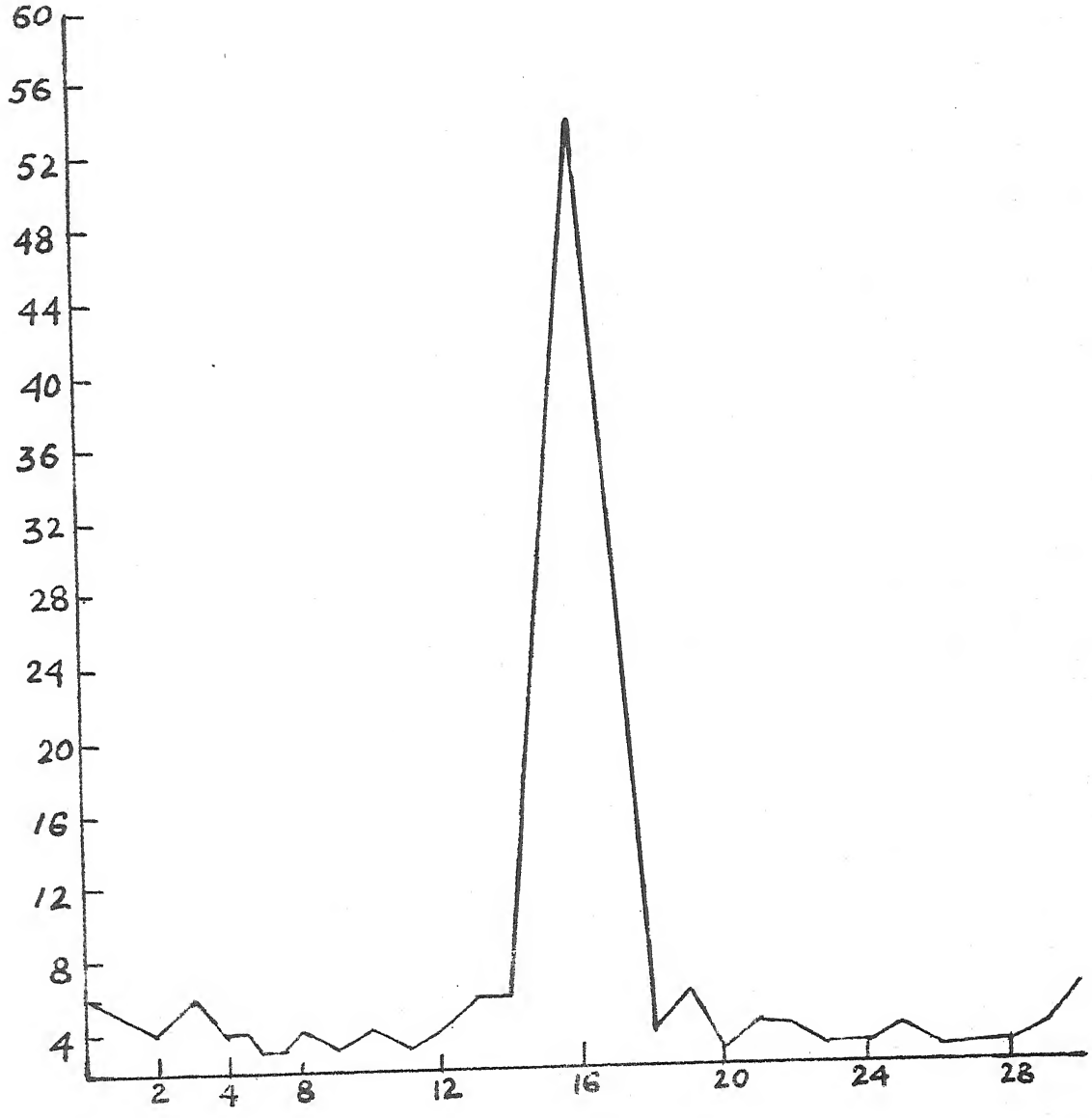
III.2 STRUCTURAL ELUCIDATION OF NEUTRAL WATER SOLUBLE POLYSACCHARIDE FROM THE SEEDS OF PHASEOLUS MUNGO

III.2.1 RESULTS AND DISCUSSION

A new water soluble polysaccharide has been isolated from the defatted seeds of P. mungo by extracting with 1% acetic acid and precipitating with excess of ethanol. The polysaccharide was repeatedly purified till the ash content reduced to minimum. The homogeneity of the polysaccharide was checked by :

(i) Fractional Precipitation ,
(ii) Zone - electrophoresis , and
(iii) Acetylation - deacetylation.

The polysaccharide was dissolved in water and separated into three fractions by fractional precipitation with different volumes of ethanol. All the three samples were analysed quantitatively by the methods of Hirst and Jones[47]. The results were essentially identical to the original polysaccharide showing the polysaccharide to be homogeneous.



Segment number

Fig. (1)

The portion of the polysaccharide was separated by zone-electrophoresis method in borate buffer (pH 9.3). After completion of the experiment, a plot of the absorbance against segments numbers showed only a single sharp peak indicating the polysaccharide to be homogeneous.(Fig. 1)

The homogeneous polysaccharide was acetylated with acetic anhydride and sodiumacetate. The acetylated product showed optical rotation, $[\alpha]_D^{25}$ + 28.5° (in chloroform, C, 0.88%). On deacetylation, it gave a polysaccharide having the same optical activity as the original one. Thus it confirmed the homogeneity of the polysaccharide.

III.2.2 The polysaccharide was slowly soluble in water, $[\alpha]_D^{25}$ + 72.2° (in water, C, 0.46), ash content 0.62%. The polysaccharide was found to be free of nitrogen, sulphur, and halogens. The methoxyl, uronide and acetyl percentages were found to be negligible.

III.3 The complete acid hydrolysis of the polysaccharide with 2% sulphuric acid followed by the paper chromatographic analysis of the hydrolysate revealed the presence of two sugars, D-galactose and D-mannose. The identity of the sugars was confirmed by their specific optical rotations, preparation of their crystalline derivatives and co-chromatography with authentic samples.

The quantitative estimation of mono-saccharide components by periodate oxidation, taking ribose as a reference sugar, showed that galactose and mannose are present in the molar ratio, 1:4 in the polysaccharide.

The graded hydrolysis of the polysaccharide with 0.05N sulphuric acid and subsequent paper chromatographic analysis of the hydrolysate, taking out at various intervals, revealed that galactose was liberated first followed by the liberation of mannose This shows that mannose units are linked together forming the backbone (main chain) of the polysaccharide and most of the galactose units are linked as terminal groups. The easy liberation of galactose units indicates that most probably they are linked to the main chain at peripheri through α-linkages.

III.d The polysaccharide was methylated first by Haworth's method using dimethyl sulphate and alkali[44] followed by Purdie's method[45] giving a methylated polysaccharide, $[\alpha]_D^{25}$ + 43.4° (in chloroform, C, 1.3%), OMe, 46.4%. The complete hydrolysis of the methylated polysaccharide and paper chromatographic analysis of the hydrolysate in solvent (A), revealed the presence of four methylated sugars. The methylated sugars were separated on a preparative scale by chromatography on Whatman No.3 filter paper. The following methylated sugars were identified.

(I) 2,6-Di-O-methyl-D-galactose;

(II) 2,3-Di-O-methyl-D-mannose;

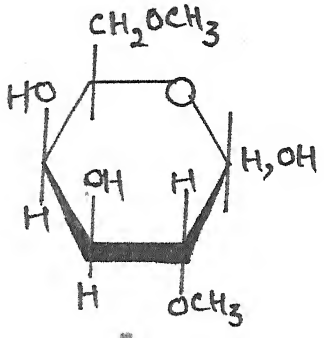
(III) 2,3,6-Tri-O-methyl-D-mannose;

and (IV) 2,3,4,6-Tetra-O-methyl-D-galactose.

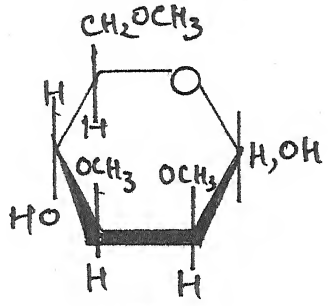
Methylated sugar, I, had $R_{2,6G}$ in solvent (A), 0.46, $[\alpha]_D^{25}$ + 80° (in water, C, 0.6%). It formed 2,6-di-O-methyl-D-galactose anilide on treatment with ethanolic aniline, m.p. 120-22°, $[\alpha]_D^{25}$ + 13° (in ethanol, C, 0.6%). On oxidation with bromine water it gave a lactone, $[\alpha]_D^{25}$ - 22° (in water, C, 1.2%)

which on treatment with phenyl hydrazine formed 2,6-di-O-methyl galactonic acid phenyl hydrazide, m.p. 133°. Thus the above observations confirmed that the methylated sugar , I, is 2,6-di-O-methyl-D-galactose.

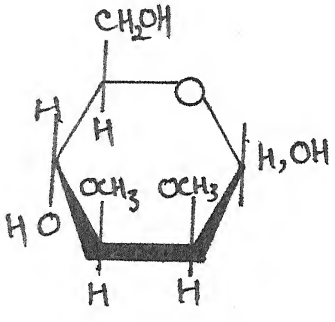
Methylated sugar, II, was obtained as a syrup, R_{DMG} in solvent (A) 0.96, $[\alpha]_D^{26}$ = 16.8° (C, 1.8% in water). It formed 1,4,6-p-nitro benzoate with p-nitrobenzoyl chloride, m.p. 191-92° $[\alpha]_D^{26}$ + 63° (in chloroform, C, 1.2%), which shows that the methylated sugar, II, is 2,3-di-methyl-D-mannose.



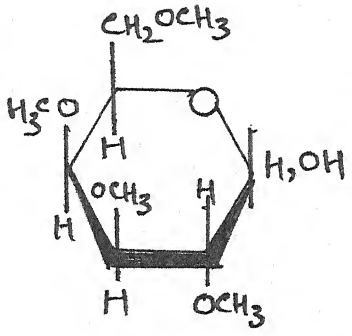
I



III



II



IV

Methylated sugar, III, R_{MG} in solvent (A), 0.83 , $[\alpha]_D^{25}$ - 12.2° (in water, C, 1.6%) formed 1,4-bis-p-nitrobenzoate, m.p. 136-38°, $[\alpha]_D^{26}$ + 32° (in chloroform, C, 0.4%). On oxidation with bromine water, it gave a lactone, which on treatment with phenyl hydrazine formed 2,3,6-tri-O-methyl-D-mannonic acid phenyl hydrazide, m.p. 129-30°. This indicates that the methylated sugar, III is 2,3,6-tri-O-methyl-D-mannose.

Methylated sugar, IV, R_{MG} in solvent (A), 0.93 , $[\alpha]_D^{25}$ + 120° (in water, C, 0.6%). On treatment with ethanolic aniline gave 2,3,4,-6-tetra-O-methyl-N-phenyl-D-galactosylamine, m.p. 188-90°. Therefore the identity of the methylated sugar IV, is established as 2,3,4,6-tetra-O-methyl-D-galactose.

The quantitative estimation of methylated sugars by the method of Hirst and Jones[46] showed that the sugars I, II, III, and IV were present in the molecular ratio, 1 : 4 : 20 : 5.

The studies indicate that galactose units in the polysaccharide occupy terminal positions as non-reducing end groups from which 2,3,4,6-tetra-O-methyl-D-galactose IV,, arises on hydrolysis of the methylated polysaccharide. A large portion of III, 2,3,6-tri-O-methyl-D-mannose (20 moles) indicates that the back-bone of the polysaccharide consists of mannose units linked through 1 → 4 linkages. Isolation of 2,6-di-O-methyl-D-galactose (1 mole) made an idea that one mole of galactose unit per repeating unit of the polysaccharide is linked at position 1, 3, and 4. Detection of 2,3-di-O-methyl-D-mannose (4 moles) shows that four mannose units in the main chain per repeating unit of the polysaccharide are linked at position -6 in addition to -1 and 4- positions.

III.5 Determination of terminal groups by periodate oxidation and subsequent titration of liberated formic acid, corresponds to 0.1036 moles of formic acid per 100 g of the polysaccharide, is supposed to consist of 30 sugar moieties of which 5 units of galactose form terminal groups. Considering such a repeating unit, the terminal groups were found 16.90% as determined by periodate oxidation studies, which is identical to that revealed by methylation studies (16.79%).

III.6 The partial acid hydrolysis of the polysaccharide followed by paper chromatographic separation on preparative scale afforded six oligosaccharides. The following oligosaccharides were detected :

1. Mannotetrose, O-β-D-mannopyranosyl-(1 → 4)-O-β-D-mannopyranosyl-(1 → 4)-O-β-D-mannopyranosyl-(1 → 4)-D-mannopyranose.
2. Mannotriose, O-β-D-mannopyranosyl-(1 → 4)-O-β-D-mannopyranosyl-(1 → 4)-D-mannopyranose.
3. Epimelibiose, 6-O-β-D-galactopyranosyl-D-mannopyranose.
4. Mannobiose, 4-O-β-D-mannopyranosyl-D-mannopyranose.
5. 6^2-α-galactosyl mannobiose, O-α-D-galactopyranosyl-(1 → 6)-O-β-D-mannopyranosyl-(1 → 4)-D-mannopyranose.
6. Galactobiose, 3-O-α-D-galactopyranosyl-D-galactopyranose.

Oligosaccharide, (1), m.p. 230-32°, $[\alpha]_D^{32}$ - 27.8° (in water, C, 1.2%), was crystallised from aqueous ethanol. It was found chromatographically pure in three solvents systems F, C and B (Page 84). The complete acid hydrolysis followed by paper chromato-

graphic analysis revealed the presence of only mannose units in the oligosaccharide. The equivalent weight, 337.3, of the oligosaccharide corresponds to a tetrasaccharide. The hydrolysis with the enzyme, emulsin and the negative rotation indicated that the mannose units in the oligosaccharide are linked through β-linkages. Partial acid hydrolysis yielded mannose, mannobiose, and mannotriose which were identified by their co-chromatography with the authentic samples. The periodate oxidation revealed the liberation of 2.12 moles of formic acid with the consumption of 6.20 moles of metaperiodate per mole of the oligosaccharide. On the basis of these experimental evidences, the oligosaccharide has been identified as O-β-D-mannopyranosyl-(1 → 4)-O-β-D-mannopyranosyl-(1 → 4)-O-β-D-mannopyranosyl-(1 → 4)-D-mannopyranose (Fig. - 2).

Oligosaccharide (2), m.p. 167-69°. $[\alpha]_D^{32}$ - 18.8° (in water C, 1.8%) was chromatographically pure in solvents (C), (G) and (F). It was shown to be monohydrate of trisaccharide on the basis of its equivalent weight , 264.8. Acid hydrolysis of the oligosaccharide yielded only mannose. The anomeric configuration of non-reducing mannose units were found to be by enzymic hydrolysis and negative rotation was found within the range of that reported for mannotriose. Partial acid hydrolysis yielded mannose and mannobiose which were identified by co-chromatography with the authentic samples. The identity was also confirmed by the periodate oxidation data which showed the liberation of 2.10 moles of formic acid with the consumption of 3.3 moles of metaperiodate per mole of sugar. Hence the oligosaccharide was identified to be O-β-D-mannopyranosyl-(1 → 4)-O-β-D-mannopyranosyl-D-mannopyranose. (Fig. - 3).

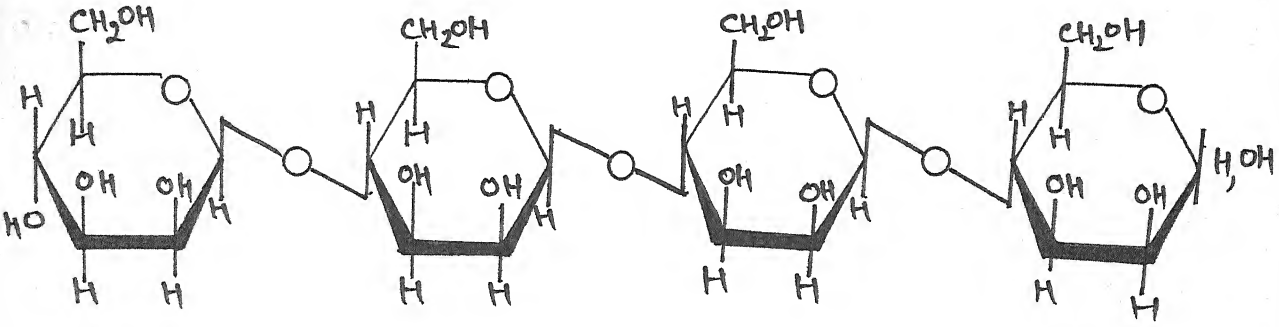


Fig-2

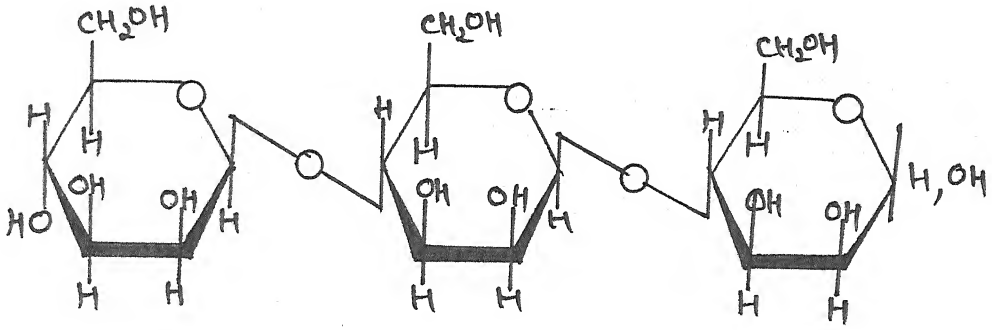


Fig-3

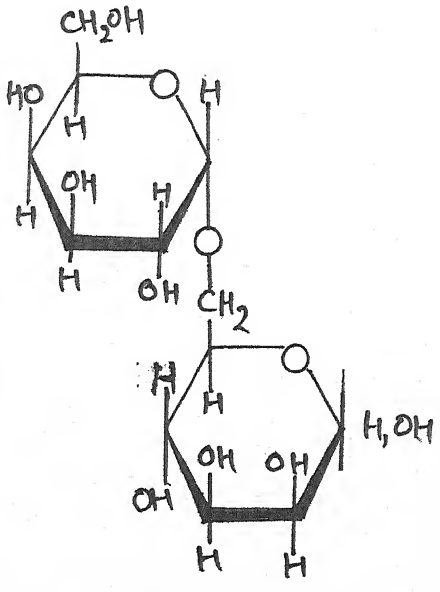


Fig-4

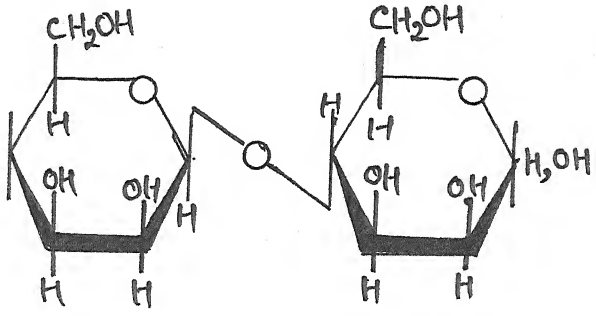


Fig-5

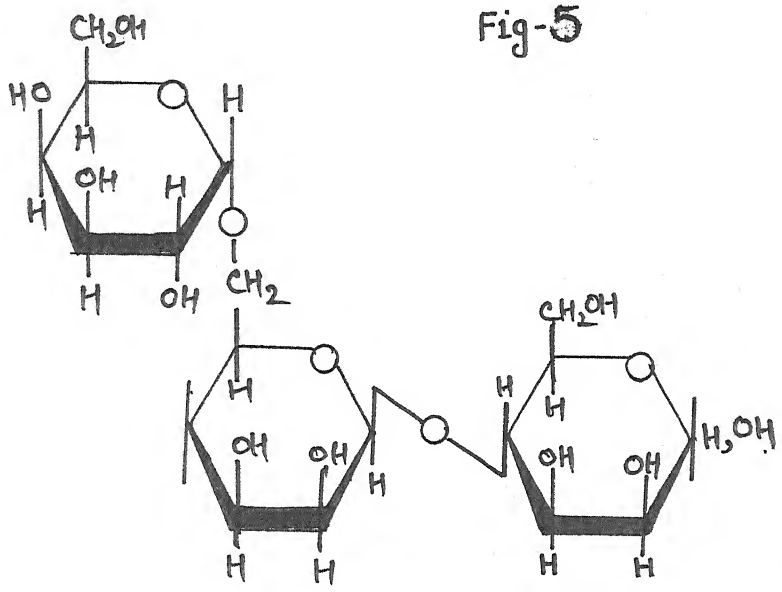


Fig-6

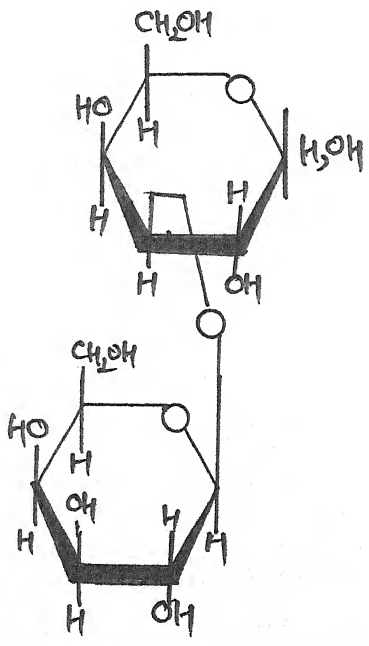


Fig-7

Oligosaccharide (3), was isolated in crystalline form having the physical constants identical with those reported for 6-O-α-D-galactopyranosyl-D-mannopyranose. It reduced Fehling's solution and Tollen's reagent having m.p. 200-01°. $[\alpha]_D^{32}$ + 130.4° (in water, C, 0.48%) and was found to be a single entity by paper chromatography in three different solvent systems (A), (B) and (C). The paper chromatographic analysis of the completely hydrolysed sugar revealed the presence of galactose and mannose. The quantitative estimation by the method of Hirst and Jones[47] showed the molar ratio to be 1:1 between the two sugars in the oligosaccharide. The equivalent weight, 174.2, showed it to be a disaccharide. The periodate oxidation studies afforded the liberation of 2.2 moles of formic acid and consumption of 3.24 moles of periodate per mole of the disaccharide. The liberation of 2.2 moles of formic acid from the disaccharide indicates that there is 1 → 6 linkage between galactose and mannose units. As the disaccharide could not be hydrolysed with emulsin, it is inferred that galactose and mannose have α -linkage between them. On the basis of above evidences, the oligosaccharide was identified to be epimelibiose, 6-O-α-D-galactopyranosyl-D-mannopyranose and identity was further confirmed by co-chromatography with an authentic sample (Fig. - 4).

Oligosaccharide (4), was also isolated in crystalline form, having the physical constants identical with those reported for 4-O-β-D-mannopyranosyl-D-mannopyranose. It reduced Fehling's solution and Tollen's reagent. The sugar on acid hydrolysis yielded only mannose while the equivalent weight of the sugar, 174.8, corresponded to a hexose disaccharide. Enzymic hydrolysis with emulsin showed the presence β-linkage between the two mannose units. The periodate oxidation showed the liberation of 2.14 moles

of formic acid with the consumption of 4.22 moles of metaperiodate per mole of the sugar. Hence the oligosaccharide was assigned the structure, 4-O-β-D-mannopyranosyl-D-mannopyranose. The identity was confirmed by co-chromatography with an authentic sample (Fig. - 5).

Oligosaccharide (5) was crystallised from ethanol, m.p. 266° - 268°, $[\alpha]_D^{32}$ + 98.8° (in water , C, 0.4%). It was shown to be a single entity by paper chromatography in solvent systems (G), (C) and (S) (page 84). It reduced Fehlings solution and Tollen's reagent The complete acid hydrolysis of sugar and subsequent paper chromatographic examination revealed the presence of galactose and mannose. The quantitative estimation by the method of Hirst and Jones[47] showed that galactose and mannose are present in the oligosaccharide in the ratio 1:2. The equivalent weight , 262.8, showed it to be a trisaccharide. The periodate oxidation studies showed the liberation of 3.18 moles of formic acid with the consumption of 6.30 moles of metaperiodate. Partial acid hydrolysis followed by paper chromatographic examination showed the presence of mannobiose and epimelibose besides galactose and mannose. Their identity was confirmed by co-chromatography with their authentic samples. The oligosaccharide was, thus identified as O-α-D-galactopyranosyl-(1 → 6)-O-β-D-mannopyranosyl-(1 → 4)-D-mannopyranose. (Fig. - 6).

Oligosaccharide (6), $[\alpha]_D^{30}$ + 132° (in water, C, 1.2%) was shown to be chromatographically pure in solvent system (G) (page 84) Acid hydrolysis showed the presence of only galactose units and its equivalent weight , 173.4, corresponded to a hexose disaccharide. It could not be hydrolysed with emulsin. The periodate oxidation showed the liberation of 1.88 moles of formic acid and the consump-

tion of 3.12 moles of metaperiodate per mole of the sugar. The oligosaccharide is, therefore identified to be 3-O-α-D-galactopyranosyl-D-galactose. (Fig. - 7).

III.B On the basis of the results obtained so far particularly from the methylation studies, graded and partial acid hydrolysis, the following valuable informations could be derived.

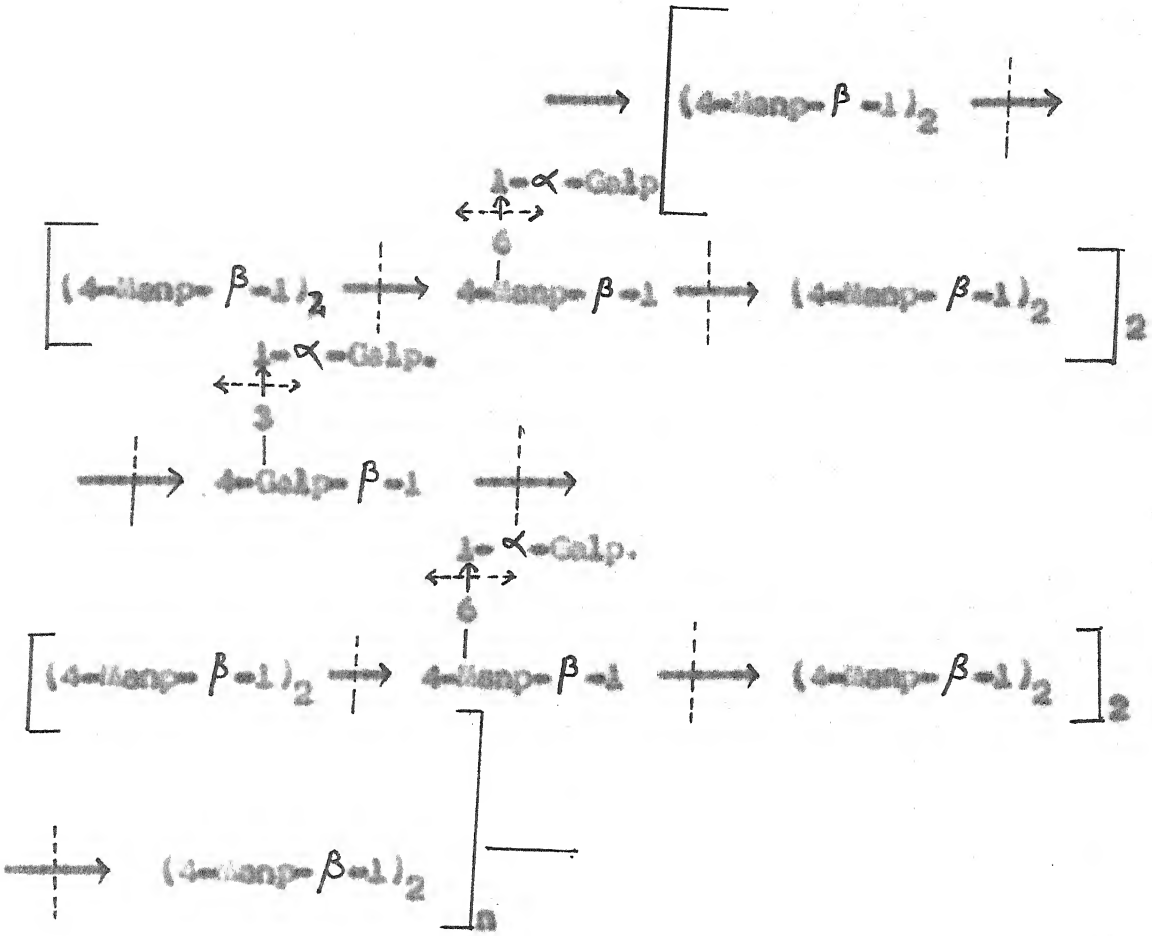
(i) The main chain of the polysaccharide consists of β-(1 → 4) linked mannose units.

(ii) One galactose unit per repeating unit of the polysaccharide is also linked in the main chain through β-(1 → 4)-linkage.

(iii) Galactose units form single unit branches linked to the main chain through α-linkages.

(iv) α-(1 → 6)-linkages between galactose and mannose units and α-(1 → 3)-linkage between two galactose units are present in the side chain only.

Taking all the experimental evidences into consideration together with the structures of different oligosaccharides, the following most probable structure has been assigned to the polysaccharide from the seeds of Phaseolus mungo.



Galp = D-galactopyranose

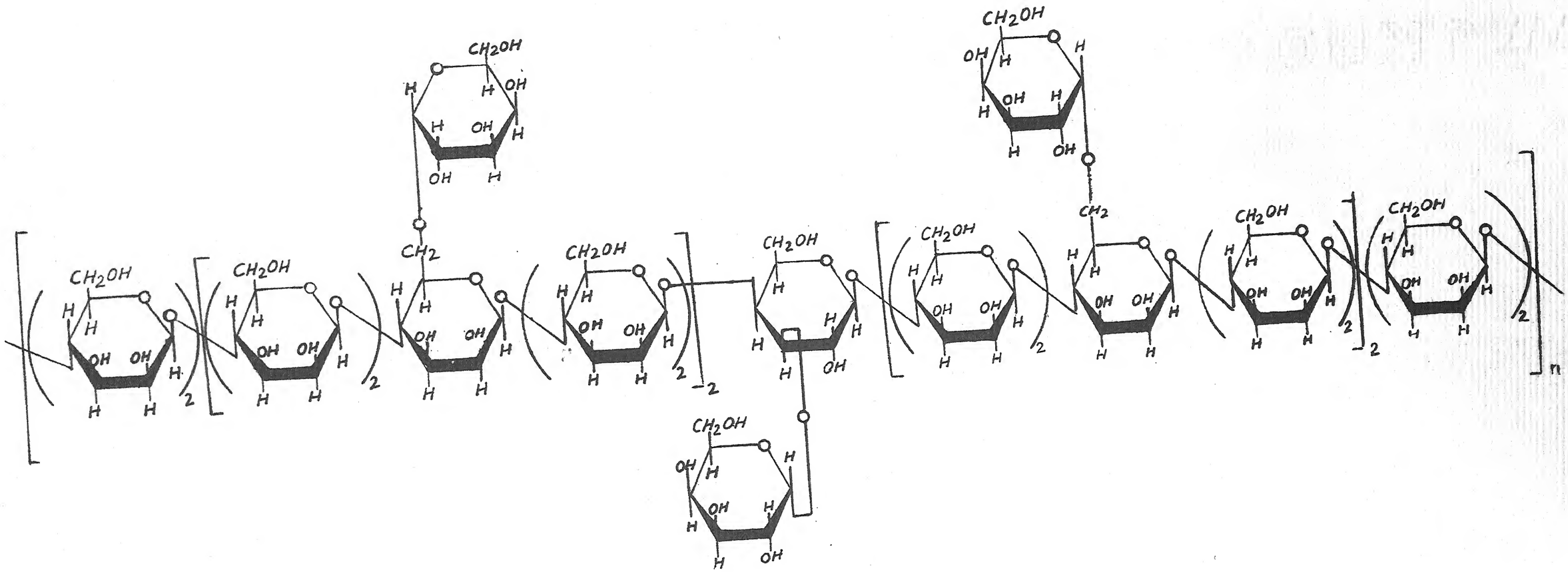
Manp = D-mannopyranose.

The above structure contains 30 units of hexose monosaccharides per repeating unit, which fully explains the formation of oligosaccharides as obtained by partial acid hydrolysis and agrees well with the analytical data of the polysaccharide. The dotted and doubly arrowed dotted lines show the probable mode of fission of linkages during the partial acid hydrolysis . The arrowed dotted lines indicate secondary hydrolysis.

The polysaccharide such as described above should consume 34 moles of metaperiodate with the liberation of 5 moles of formic acid per repeating unit of 30 sugar units. The actual consumption

of periodate and liberation of formic acid have been determined to be 34.4 moles and 5.04 moles respectively per repeating units of polysaccharide which are in close agreement to the calculated values.

Possibility of these similar structures can not be completely ruled out but they are less probable because the xxxxx formation of oligosaccharide as obtained in the present case might not be possible.



STRUCTURE OF POLYSACCHARIDE FROM THE SEEDS OF PHASEOLUS MUNGO

III.9 EXPERIMENTAL

Experimental techniques were same as described on page ?
Paper chromatography was performed at room temperature by descending technique on Whatman No.1 filter paper unless stated otherwise using following solvent systems :

(A)	n-Butanol - ethanol - water	(5:1:4)[49,50]
(B)	n-Butanol - acetid acid - water	(4:1:5)[51]
(C)	n-Butanol - iso-propanol - water	(11:1:5)[52]
(D)	Benzene - ethanol - water	(169:47:15)[53]
(E)	Butanone - water	(10:1)[54]
(F)	Ethyl acetate - pyridine - water	(10:4:3)[55]
(G)	Ethyl acetate - pyridine - water	(2:1:2)[56]
(H)	n-Butanol - ethanol - water	(40:11:19)[57]
(I)	n-Butanol - pyridine - water	(6:4:3)[58]

The spot were located by spraying the chromatgram with aniline hydrogen phthalate[59] and heating it at 110-20° for 10-15

minutes. Spectrometric determination were carried out by a modification of phenol-sulphuric acid method[60]. Klett-Summerson photoelectric colorimeter was used for measuring the absorbance.

III.10 ISOLATION OF THE POLYSACCHARIDE

The dried and crushed seeds (2 Kgs) were extracted successfully with petroleum ether (60-80°) and ethanol. The polysaccharide extracted from the extracted seeds by repeating the process as given on page 31. A colourless fibrous precipitate of the crude polysaccharide was obtained. It was filtered, washed with absolute ethanol and dried in vacuum at room temperature (39 g. ash 2.12%).

III.9 PURIFICATION

The dried crude polysaccharide was dissolved in distilled water (2 litres) containing 1% acetic acid with constant stirring The solution was filtered and was added very slowly to ethanol (8 litres) with constant stirring and kept over-night. The precipitated polysaccharide was filtered and the above process was repeated four times, to get a white fibrous mucilage. (26 g. ash 0.6).

III.11 HOMOGENEITY OF THE POLYSACCHARIDE

The homogeneity of the polysaccharide was checked by following methods.

III.11.1 (a) Fractional Precipitation

The pure mucilage (4 g) was fractionally precipitated into

two fractions (Fraction I and Fraction II). Both the fractions along with the original polysaccharide were hydrolysed and quantitatively estimated by the usual way as described on page 32. The ratio of galactose and mannose in both the fractions was found almost the same (1:4) indicating the purified polysaccharide to be homogeneous.

III.11.2 (b) Zone - Electrophoresis

Polysaccharide (300 mg) was taken for zone electrophoresis and similar procedure was adopted as described on page 34.

The corrected absorbance readings (Table - 1) so obtained were plotted against the distance from the anode , that is segment number which showed only one sharp peak indicating the polysaccharide to be homogeneous.

III.11.3 (c) Acetylation and Deacetylation

The pure polysaccharide (1.5 g) was mixed thoroughly with anhydrous sodium acetate (10 g) and the mixture was suspended in acetic anhydride (30 mls) and further process was repeated as on page . The acetylated polysaccharide (1.1 g) was obtained having $[\alpha]_D^{25}$ + 28.5° (in chloroform, C, 0.68%).

The dried acetylated polysaccharide (0.8 g) was dissolved in acetone (30 ml) and 50% potassium hydroxide solution (30 ml) was added to it. The acetylation was carried in the usual manner[48] as given on page 33 . The deacetylated polysaccharide (0.3 g) having $[\alpha]_D^{25}$ + 71.5° (in water, C, 0.61%).

TABLE no. 1

Segment No.	Klett reading of eluate	Blank Klett reading	Corrected Klett reading	Absorbance
1	25	22	3.0	0.006
2	25	23	2.0	0.004
3	24	21	3.0	0.006
4	24	22	2.0	0.004
5	24	22	2.0	0.004
6	23	22	1.0	0.002
7	22	21	1.0	0.002
8	23	21	2.0	0.004
9	23	22	1.0	0.002
10	24	22	2.0	0.004
11	23	22	1.0	0.002
12	24	22	2.0	0.004
13	24	21	3.0	0.006
14	24	21	3.0	0.006
15	39	22	17.0	0.034
16	49	22	27.0	0.054
17	38	22	16.0	0.032
18	23	21	2.0	0.004
19	25	22	3.0	0.006
20	23	22	1.0	0.002
21	24	22	2.0	0.004
22	24	22	2.0	0.004
23	23	22	1.0	0.002
24	23	22	1.0	0.002
25	24	22	2.0	0.004
26	23	22	1.0	0.002
27	23	22	1.0	0.002
28	24	23	1.0	0.002
29	25	23	2.0	0.004
30	25	21	4.0	0.008

Absorbance was measured on 5 ml portion of coloured solution.

$$\text{Absorbance} = \frac{2 \times \text{Klett reading}}{1000}.$$

The original polysaccharide, $[\alpha]_D^{25}$ + 72.2° (in water, C, 0.6%) and the polysaccharide obtained after deacetylation had almost the identical specific rotations indicating the homogeneity of the polysaccharide.

III.12 ASH CONTENT

The dried polysaccharide (0.2 g) was ignited in a silica crucible previously heated to a constant weight. After ignition, the crucible was cooled in a desiccator and weighed. From the weight of residue (0.0014 g), the ash content was calculated to 0.62%.

III.13 PHYSICAL AND CHEMICAL EXAMINATION

It was a fibrous white powder, very light in weight, slowly soluble in water, $[\alpha]_D^{25}$ + 72.2° (in water, C, 0.6%). For the purpose of optical rotation, the solution was filtered through a sintered funnel to get a clear solution and the amount of polysaccharide in the solution was determined colorimetrically. The polysaccharide was found to be free of nitrogen, sulphur, and halogens. On treatment with Fehling's solution, it formed an insoluble copper complex but did not reduce it.

III.14 EXAMINATION OF FREE SUGARS

The polysaccharide was examined for free sugars by applying three spots of its solution in water on a strip of Whatman No.1 filter paper (15 x 45 cms) and developed in solvent (A) as described on page 36. The three spray reagents naphthoresorcinol and

trichloroacetic acid[61], aniline hydrogen phthalate[57] and silver nitrate in acetone followed by ethanolic sodium hydroxide[62] on three different strips of above paper showed no spot, hence it showed that the polysaccharide did not contain any free sugar.

III.15 METHOXYL GROUPS DETERMINATION

The percentage of methoxyl groups was determined by the method of Belcher, Fildes and Nutten[63] and was found to be negligible. (0.84%).

III.16 ACETYL GROUPS DETERMINATION

The method by Belcher and Godbert[64] was followed for the determination of acetyl group percentage with and without mucilage which was found insignificant (0.96%).

III.17 URONIDE CONTENTS DETERMINATION

The uronide contents were found to be negligible by the semi-micro method of Baker, Foster, Siddiqui and Stacey[65].

III.18 HYDROLYSIS OF POLYSACCHARIDE AND DETERMINATION OF MONOSACCHARIDE

The purified mucilage (1.5 g) was dissolved in 2N sulphuric acid (100 ml) and was hydrolysed on a water-bath for about 24 hours. The hydrolysate was neutralised with barium carbonate, filtered and concentrated under reduced pressure. The hydrolysate was examined

paper chromatographically for monosaccharides.

III.18.1 (a) Paper Chromatography

The spots of hydrolysate were applied on two sheets of Whatman No.1 filter paper. The papers were developed separately in solvents(A) and (B) by descending unidimensional technique. The chromatograms were air-dried and sprayed with aniline hydrogen phthalate. On heating them in an oven at 120°, each chromatogram showed two spots. The R_g and R_G values of the two spots corresponded to D-galactose and D-mannose as given in the following Table.

TABLE - 2

Sugar identified	Solvent (A)		Solvent (B)	
	R_G found	R_G given 66	R_g found	R_g given 51
D-Galactose	0.08	0.07	0.16	0.16
D-Mannose	0.12	0.11	0.20	0.21

G = 2,3,4,6-Tetra-O-methyl-D-galactose.

The identity of the two sugars was further confirmed by co-chromatography with authentic samples of the sugars in the same solvent.

XII.16.2 (b) Column Chromatography

A portion of hydrolysate was dissolved in a small amount of aqueous methanol (1:1) and adsorbed over a column of cellulose (2 x 35 cms.). The column was left over-night and the separation was effected with solvent (A). Fractions amounting to 10 ml were collected and checked by paper chromatography with authentic samples of D-galactose and D-mannose in solvent (B). The fractions 1-12 containing same sugar were combined together and concentrated to give D-mannose. It was recrystallised from aqueous methanol, $[\alpha]_D^{25}$ + 12.6° (in water, C, 1.6 of per 100 ml of solution). The following two derivatives were prepared.

(i) D-mannose phenyl hydrazone

Found	Given (Lit.)[67]
m.p. 192-94°	199-200°.

(ii) D-mannose p. n. glycosylamino benzoic acid

The derivative was prepared according to the recent method of Ellis[66].

Found	Given (Lit.)[66]
m.p. 180-81°	182°.

The fractions 20-28 were mixed and concentrated to give D-galactose. It was recrystallised from aqueous methanol. $[\alpha]_D^{25}$ + 79.2° in water, C, 0.5 g per 100 ml of solution). The following derivatives were prepared :

(i) D-Galactose phenyl hydrazine

	Found	Given (lit.)[69]
m.p.	193-94°	194-95°.

(ii) N-p-Nitrophenyl - D- Galactopyranose

In a micro-test tube , galactose (50 mg), p-nitroaniline (50 mg), one drop of glacial acetic acid and four drops of methanol -water (8:1 v/v) were taken. The mixture was boiled for 8 minutes and kept over-night in a refrigerator. The crystalline product was filtered, washed with cold ethanol, ether and dried in vacuum. It melted at 218-19° after recrystallisation from methanol. Lit.[70], m.p. 219°.

III.18.3 (c) Thin - Layer Chromatography

The plates were prepared from slurry of silica gel G in 0.1M solution of boric acid and the spots of hydrolysate along with benzene : acetic acid : methanol (1 : 1 : 3)[71] and air-dried. These plates were sprayed with aniline hydrogen phthalate reagent on heating them at 120° in an electric oven two spots corresponding to D-galactose and L-rhamnose were observed.

III.19 QUANTITATIVE ESTIMATION OF MONOSACCHARIDE

The polysaccharide (200 mg) was hydrolysed with 2N sulphuric acid (35 ml) for 24 hours on a boiling water-bath and neutralised with barium carbonate. Ribose (20 mg) was added to the hydrolysate. The hydrolysate was applied on Whatman No. 1 filter paper along with the guide spots. After developing in solvent (C), the strips corresponding to the sugars were cut with the help of guide spots and eluted. The eluate was oxidised with periodate and the

quantity of the monosaccharide estimated as described on page •

Table - 3

Sugar	Volume of alkali* used (in ml)			Corresponding amount of sugar (in mg)		
	A	B	C	A	B	C
Galactose	3.36	4.12	2.76	0.97	1.21	0.80
Mannose	13.60	16.98	11.16	3.92	4.89	3.21
Ribose	1.62	2.04	1.42	0.48	0.61	0.42

* Strength of sodium hydroxide = N/124.8.

Assuming complete recovery of D-ribose the above results indicate that in the polysaccharide D-galactose and D-mannose are in the molar ratio of 1:4.

III.20 GRADED HYDROLYSIS[73] OF THE POLYSACCHARIDE

The polysaccharide (100 mg) was dissolved in 0.05N sulphuric acid (10 ml). The hydrolysis carried out over a boiling water-bath . The hydrolysate, taken out at various intervals, were examined chromatographically, without removal of sulphuric acid using solvent (B) for the purpose of irrigation of the paper. Results are given in the Table - 4.

TABLE - 4

Time (in minutes)	Sugar identified	No. of other spots
10	-	-
15	Galactose (Faint)	Two spots (very faint)
30	Galactose	Three spots (Faint)
45	Galactose	Three spots (clear)
60	Galactose	Two spots (clear)
120	Galactose + Mannose (very faint)	Two spots (clear)
180	Galactose + Mannose	Same as above
240	Galactose + Mannose	Same as above

D-Galactose was found to liberate first followed by the liberation of D-mannose. The easy release of D-galactose leads to the conclusion that most of it is present as terminal group and not in the main chain of the polysaccharide.

III.21 METHYLATION OF THE POLYSACCHARIDE

The polysaccharide (8 g) was methylated first by the method due to Parikh, Ingle and Bhide[44] followed by Purdie's method[45] as usual described on page 43.

The partly methylated product was brownish mass (6.80 g). $-OCH_3$, 36.4% ; $[\alpha]_D^{25}$ + 54.2° (in chloroform, C, 1.5 per 100 ml of solution) . The partly methylated polysaccharide was further methylated by Purdie's method as given on page 43 . The fully methylated polysaccharide was obtained as a deep brown coloured product (5.2 g). found $-OCH_3$, 46.4%. $[\alpha]_D^{25}$ + 43.4° (in chloroform,

1.3 g per 100 ml of solution) .

III.22 HYDROLYSIS OF THE METHYLATED POLYSACCHARIDE AND IDENTIFICATION OF METHYLATED SUGARS

The hydrolysis of methylated polysaccharide was carried by slight modification of method due to Bouveng et-al[74]. The methylated polysaccharide (100 mg) was dissolved in 85% formic acid (20 ml) and rest of the process was carried out as described on page 44.

After separation on Whatman No.1 filter paper in solvent (A), the chloroform chromatogram of syrup showed four spots after spraying with aniline hydrogen phthalate and drying at 120°. The R_{TMG} value of each methylated sugar was calculated in solvent (A) and was compared with that, given in literature as shown in the following Table -5 .

TABLE - 5

Methylated sugars identified	Solvent (A) 49,30,91	
	R_{TMG} found	R_{TMG}
2,6-Di-O-methyl-D-galactose	0.46	0.44
2,3-Di-O-methyl-D-mannose	0.36	0.34
2,3,6-Tri-O-methyl-D-mannose	0.80	0.81
2,3,4,6-Tetra-O-methyl-D-galactose	0.90	0.88

III.23 QUANTITATIVE ESTIMATION OF METHYLATED SUGARS

III.23.1 The methylated polysaccharide (200 mg) was hydrolysed as given above. To the hydrolysate glucose (40 mg) was added and

then neutralised with barium carbonate.

The chromatograms were developed by the descending method using solvent (D) as described on page 45.

The sugars were estimated by alkaline hypoiodite method as given on page 46 . The results obtained are given in the Table - 6.

TABLE - 6

Fraction & Sugar	Volume of 0.1N hypo used (in ml)			Corresponding amount of sugars (in mg)		
	A	B	C	A	B	C
A. 2,6-Di-O-methyl-D-galactose	0.16	0.22	0.18	0.152	0.209	0.171
B. 2,3-Di-O-methyl-D-mannose	0.64	0.88	0.72	0.608	0.836	0.684
C. 2,3,6-Tri-O-methyl-D-mannose	3.00	4.08	3.34	3.060	4.161	3.406
D. 2,3,4,6-Tetra-O-methyl-D-galactose	0.68	1.00	0.78	0.741	1.090	0.850
E. Glucose	1.26	1.76	1.42	1.134	1.584	1.278

The above results corresponded to an average molar ratio between A, B, C and D as 1 : 4 : 20 : 5. The methylated sugars were calculated as the methyl ethers of anhydrohexose units i.e. $C_{10}H_{18}O_5$, $C_9H_{16}O_5$, and $C_8H_{14}O_5$ for tetra-, tri-, and di-O-methyl sugars respectively. An average recovery of the methylated polysaccharide was found to be 99.90% assuming 100% recovery of glucose.

III.23.2 CHARACTERISATION OF METHYLATED SUGARS

The methylated polysaccharide was hydrolysed according to the method of Gaegg and Lindberg[76] as described on page 47.

The mixture of different methylated sugars was resolved into five fractions on Whatman No.3 filter paper using solvent (D) Strips containing different individual methylated sugars were eluted with water. The eluates were concentrated seperately under reduced pressure and marked as fractions, I, II, III, IV and IV.

III.23.3 Fraction I

Solid, R_{TMG} in solvent (A), 0.46 , found : OMe, 29.16% calculated for di-methyl hexose, OMe, 29.03%. It was pink brown spot on spraying with p-aniasidine hydrochloride, m.p. 116-18°. $[\alpha]_D^{25}$ + 80° (in water, C, 0.6%). Lit.[77], m.p. 119-20°. $[\alpha]_D$ + 46° (in water, C, 0.4%) → + 84° (equilibrium value). It gave 2,6-di-O-methyl-D-galactose anilide on treatment with ethanolic aniline, m.p. 120-22°. $[\alpha]_D^{25}$ + 18° (in ethanol, C, 0.6%, Lit.[78] m.p. 119-20°. $[\alpha]_D^{17}$ + 15° (in ethanol, C, 0.7%).

The solid (150 mg) was oxidised with bromine water and the product, after neutralisation with silver carbonate, was distilled to give a syrup $[\alpha]_D^{25}$ - 22° (in water, C, 1.2%). Lit.[79] for 2,6-di-O-methyl-γ-lactone, $[\alpha]_D^{17}$ - 49° (initial) → (in water, equilibrium, C, 1.0%). The lactone (50 mg) was allowed to react with phenyl hydrazine (1 mole) in boiling ether for 15 minutes on removal of solvent and heating at 95° for two hours a crystalline product was obtained, m.p. 138°. Lit.[79] for

2,6-di-O-methyl galactonic acid phenyl hydrazide, m.p. 140°.

III.23.4 Fraction II

Syrup, R_{TMG} in solvent (A), 0.36, found : OMe, 29.44%, calculated for dimethyl : OMe, 29.01%. $[\alpha]_D^{26}$ - 16.8° (in water, C, 1.0%), Lit.[80], di-O-methyl-D-mannose, $[\alpha]_D$ - 16.0° (water).

The sugar (100 mg) was dissolved in pyridine. It was finally washed with water and dissolved in chloroform. The insoluble portion was filtered out and the solvent from the filtrate was evaporated in a vacuum desiccator. The crude product was recrystallised from ether, m.p. 191-92°, $[\alpha]_D^{26}$ + 63.8° (in chloroform, C, 1.2%). Lit.[73], for 1,4,6-p-nitrobenzoate of 2,3-di-O-methyl -D-mannose, m.p. 194° and $[\alpha]_D$ + 65° (chloroform).

III.23.5 Fraction III

Syrup, R_{TMG} in solvent (A), 0.83, Found : OMe, 41.1%, calculated for tri-methyl hexose : OMe, 41.9%. $[\alpha]_D^{25}$ - 12.2° (in water, C, 1.6 g per 100 ml of solution), Lit.[82], for 2,3,6-tri-O-methyl-D-mannose, $[\alpha]_D$ - 10° (in water).

The syrup (150 mg) was dissolved in dry pyridine (6 ml) and treated with p-nitrobenzoyl chloride (500 mg) for 45 minutes at 60-70° and left over-night at room temperature. A saturated solution of sodium bicarbonate was added drop-wise until no effervescence occurred. After adding water (15 ml), the product was extracted with chloroform. The extract was dried over sodium sulphate, excess of solvent was taken off in vacuum and crystallised

from petroleum ether, m.p. 186-88°, $[\alpha]_D^{25}$ + 32° (in chloroform, C, 0.4 g per 100 ml of solution), Lit.[83,84] for 1,4-bis-p-nitrobenzoate of 2,3,6-tri-O-methyl-D-mannose, m.p. 187-88° and $[\alpha]_D$ + 33.0°. The syrup (150 mg) was oxidised with bromine water and the product crystallised from acetone-petroleum ether, m.p. 81-82°, Lit.[53], for 2,3,6-tri-O-methyl-γ-(+)mannolactone, m.p. 82-83°. The lactone (75 mg) was boiled under reflux in alcohol with phenyl hydrazine (45 mg). It was then refluxed with little amount of animal charcoal in ethanol and filtered. On cooling, a crystalline product was obtained which was recrystallised from ethanol, m.p. 129-30°, $[\alpha]_D^{25}$ - 18.6° (in water, C, 0.8%), Lit.[86], for 2,3,6-tri-O-methyl-D-mannonic acid phenyl hydrazide, m.p. 131°, $[\alpha]_D$ - 20° (in water).

III.23.6 Fraction IV

Solid, R_{TMG} in solvent (A), 0.90, found : OMe, 51.8%, calculated for tetramethyl hexose, OMe, 52.54%, $[\alpha]_D^{25}$ + 130° (in water, C, 0.6%); Lit.[87,88,89], for 2,3,4,6-tetra-O-methyl-D-galactose, $[\alpha]_D^{16}$ + 142° → + 117° (equil.) in water (C, 1.1%), m.p. 70-72°. It gave red colour with aniline hydrogen phthalate. Its treatment with alcoholic aniline gave 2,3,4,6-tetra-O-methyl-N-phenyl-D-galactosylamine, m.p. 188-90°.

III.24 PERIODATE OXIDATION OF THE POLYSACCHARIDE

III.24.1 (a) Liberation of Formic Acid[90] & Estimation of End Groups

The polysaccharide (500 mg) was dissolved in water (50 ml) and in the solution, potassium chloride (0.5 g) and 0.25M sodium

metaperiodate (60 ml) were added. The volume was made upto 140 ml with water. In a blank experiment potassium chloride (0.5 g) and 0.25% sodium metaperiodate (60 ml) were diluted to 140 ml with water. The oxidation was carried out in dark at room temperature as described on page 50. The aliquots of 5 ml were taken and were titrated for liberated formic acid against N/102.5 sodium hydroxide solution using methyl red as indicator. Results are given in Table - 7.

The data shows that 0.1038 mole of formic acid was liberated (72 hours) per 100 g of the polysaccharide. The amount of formic acid liberated (72 hours) corresponds to 16.80% of anhydrous hexose units present as end groups. The titre values of alkali at 48, 60, and 72 hours indicated that one mole fo formic acid was liberated per 1225 g, 1064 g, and 963.3g of the polysaccharide respectively.

Table - 7

Time (in hours)	Volume of alkali used (in ml)	Formic acid observed (in mg)	Total formic acid liberated
8	0.68	0.395	11.057
16	1.02	0.437	12.815
24	1.16	0.520	14.574
36	1.32	0.592	16.584
48	1.30	0.673	18.846
60	1.72	0.771	21.613
72	1.90	0.832	23.675
96	1.90	0.832	23.675

III.24.2 (b) Consumption of Sodium Metaperiodate[89]

The polysaccharide (220 mg) was dissolved in water (70 ml) to which 0.25M sodium metaperiodate (40 ml) was added and the total volume was made upto 120 ml with water. A blank was also prepared with 0.25M sodium metaperiodate (40 ml) diluted to 120 ml with water. The periodate oxidation was carried out at room temperature as described on page 52. The liberated iodine from 2 ml aliquots of mixture and blank were titrated at various intervals against 0.0404N sodium thiosulphate solution using starch as indicator. The readings with the polysaccharide were substracted from the corresponding readings of controlled experiment to get the titre values. The results are given in Table - 8.

TABLE - 8

Time (in hours)	Hypo used (in ml)	Periodate consumed (in mg)	Total periodate consumed (in mg)
8	1.04	4.405	269.70
16	1.06	4.668	280.11
24	1.14	4.928	295.67
36	1.18	5.100	306.05
48	1.24	5.360	321.60
60	1.36	5.879	352.74
72	1.44	6.224	373.48
84	1.46	6.311	378.67
96	1.46	6.311	378.67

The amount of periodate consumed (84 hours) corresponds to the consumption of 0.7077 mole of periodate per 100 g of the polysaccharide. After 96 hours periodate oxidised solution (10 ml)

was hydrolysed with 2% sulphuric acid (page 37). The hydrolysate examined paper chromatographically for the presence of D-galactose and D-mannose but the chromatogram did not indicate the presence of any of the two sugars.

III.2b PARTIAL ACID HYDROLYSIS OF POLYSACCHARIDE

The polysaccharide (6 g) was suspended in water (500 ml) in a three necked flask, and stirred mechanically and the same procedure was adopted as described on page 53.

III.2b.1 Examination of the Precipitate

The precipitate was hydrolysed and identified similarly as described on page 54. The chromatograms showed three spots corresponding to R_g values of D-galactose, and D-mannose which were confirmed by co-chromatography with their authentic samples.

III.2b.2 Examination of the Hydrolysate

Paper chromatographic analysis of the hydrolysate over Whatman No.1 filter paper using solvents(A) and (B) and aniline hydrogen phthalate as a spraying reagent produced nine spots thereby indicating the presence of nine sugars.

III.2b.3 Separation of Oligosaccharides

The syrup was dissolved in minimum quantity of water. It was separated by paper chromatography as described on page . The sugars were crystallised from ethanol and six fractions of

oligosaccharides and two fractions of monosaccharides were obtained.

III.2B.4 Examination of Fraction I and Identification of Mannotetraose

The fraction was recrystallised from aqueous ethanol m.p. 230-32° and $[\alpha]_D^{32}$ = 27.8° (in water, C, 1.2 g per 100 ml of solution). R_{Man} , 0.12, 0.02, and 0.09 in solvents (F), (C), and (B) respectively. It reduced Fehling's solution and ammoniacal silver nitrate.

The sugar was hydrolysed with 2N sulphuric acid, neutralised with barium carbonate and filtered. The filtrate was concentrated and examined by paper chromatography using solvents (A) and (C). The chromatogram indicated only one spot, corresponding to R_g value of mannose. Thus sugar consists of only mannose units. The equivalent weight of sugar was determined by hypoiodite method[46] and was found to be 337.5 which corresponded to a tetrasaccharide.

The periodate oxidation of the oligosaccharide showed the consumption of 6.2 moles of the ~~oligosaccharide~~ metaperiodate with the liberation of 2.12 moles of formic acid per mole of the oligosaccharide. The oligosaccharide was completely hydrolysed with emulsin suggesting β-glycosidic linkages in the molecule.

All the above results indicate that the oligosaccharide is β-Manp-1→(4-β-Manp-1)$_2$ → 4-Manp. The identification of the sugar is well supported by its constants found and reported in

literature shown in the following Table - 9.

Table - 9

Constants	Found	Reported	References
m.p.	230-32°	232-34° and 231.5-32°	(88, 91, 92)
Optical rotation	$[\alpha]_D^{32}$ - 27.8°	$[\alpha]_D$ - 31° and -31.6° → 28.7°	(88, 92)
R_{Man} in solvent (F)	0.12	0.11	(88)
R_g in solvent (G)	0.17	0.15	(88)

III.29.5 Examination of Fraction II and Identification of Mannotriose

R_{Man} . 0.09 and R_{Glu} . 0.34 in solvents (C) and (G) respectively, R_{Glu} . 0.22 in solvent (F). The sugar was crystallised from ethanol, m.p. 164-66°, $[\alpha]_D^{32}$ - 18.8° (in water, C, 1.8). It reduced Fehling's and Tollen's reagents.

The complete acid hydrolysis with 2% sulphuric acid, subsequent neutralisation with barium carbonate and examination by paper chromatography with an authentic sample only one monosaccharide , D-mannose was obtained. The equivalent weight of the sugar was found to be 264.8 by hypoiodite method[46]. Partial acid hydrolysis of sugar with 0.5% hydrochloric acid at 100° for 10 minutes resulted in formation of mannose and mannobiose which were identified by co-chromatography with their authentic samples.

Periodate oxidation of the sugar revealed that 2.10 moles of formic acid were liberated and 3.3 moles of periodate were consumed per mole of the sugar. The sugar was completely hydrolysed with emulsin suggesting that mannose units are linked through β-glycosidic linkages. On the basis of above results the sugar was identified to be mannotriose, β-Manp-1 → 4-β-Manp-1 → 4Manp which was further confirmed by its physical constants as shown in Table 10.

TABLE - 10

Constants	Found	Reported	References
m.p.	164-66°	133 - 139° and 214-15°(anhydrous)	(91,93,95, 96,97)
Optical rotation	$[\alpha]_D^{32}$ - 18.8°	$[\alpha]_D$ - 15° — -26°	(98)
R_{Glu} in Solvent (G)	0.34	0.33	(55,96)
and Solvent (F)	0.22	0.22	

III.25.6 Examination of Fraction III and Identification of *M.imelibiose*

R_{Man} 0.16, 0.23, and 0.37 in solvents (A), (B) and (C) respectively. The sugar was recrystallised from ethanol, m.p. 200-01°, $[\alpha]_D^{32}$ + 120.4° (in water , C, 0.46 g per 100 ml of solution).

Acid hydrolysis of the sugar whith 2% sulphuric acid and neutralisation of the hydrolysate with barium carbonate followed

by paper chromatographic analysis with solvent (C) revealed the presence of galactose and mannose in the sugar which was further confirmed by co-chromatography with authentic samples. The quantitative estimation by the method of Hirst and Jones[47] showed the molar ratio to be 1:1 between the two sugars in the oligosaccharide.

The equivalent weight , as determined by hypoiodite method[46] was found to be 174.2. The periodate oxidation studies corresponded to the consumption of 5.24 moles of metaperiodate and liberation of 3.2 moles of formic acid per mole of the oligosaccharide. Thus there is 1 → 6 linkage between galactose and mannose units. As the oligosaccharide could not be hydrolysed with emulsin, it was inferred that galactose and mannose have α-linkage between them.

On the basis of above evidences the oligosaccharide was identified as epimelibiose, 6-O-α-D-galactopyranosyl-D-mannopyranose. Its identity was further confirmed by preparing its osazone, m.p. 173° and co-chromatography with an authentic sample. The observed constants of the sugar were compared with those reported in literature as shown in Table - II.

TABLE - II

Sugar or derivative	Constant	Found	Reported	References
Spimelibiose	m.p.	200-01°	201-02° & 202-03°	(92, 99)
Spimelibiose	Optical rotation	$[\alpha]_D^{32}$ + 120.4° (in water)	$[\alpha]_D$ + 120.9° & $[\alpha]_D$ + 120.9° → 124.6° (in water)	(99,100)
Spimelibiose	R_{Glu} in solvent (C)	0.60	0.59	(36)
Osazone	m.p.	173°	175-76°	(100)

III.2B.7 Examination of Fraction IV and Identification of Mannobiose

R_{Man} in solvents (A), (B), and (C) were found to be 0.27, 0.46 and 0.33 respectively. The sugar was recrystallised from methanol, m.p. 202°, $[\alpha]_D^{30}$ - 10.2° (in water, C, 1.2 g. per 100 ml of solution).

Acid hydrolysis with 2% sulphuric acid, followed by neutralisation with barium carbonate and subsequent examination by paper chromatography showed the presence of mannose units only. The equivalent weight was determined by hypoiodite method[48] and was found to be 174.8.

The periodate oxidation studies showed the consumption of 4.22 moles of periodate with the liberation of 2.14 moles of

formic acid per mole of the sugar. The sugar was completely hydrolysed with emulsin showing the presence of β-linkage between the mannose units which was also confirmed by the negative optical rotation of the sugar.

Thus the oligosaccharide is a disaccharide compose of D-mannose units linked through β-glycosidic linkage. The sugar was identified to be mannobiose, 4-O-β-D-mannopyranosyl-D-mannopyranose, which was confirmed by preparing the osazone derivative , m.p. 204° and co-chromatography with an authentic sample.

The constants of sugar are given in Table - 12.

TABLE - 12

Sugar or derivative	Constant	Found	Reported	References
Mannobiose	m.p.	202°	202-04°	(33,37,81, 91,92,94)
Mannobiose	Optical rotation $[\alpha]_D^{30}$	- 10.2°	$[\alpha]_D$ - 7° → -9°	(33,81,91, 92,97)
Mannobiose	R_{glu} in solvents(F)	0.52	0.52	(33,36)
	& (G)	0.67	0.65	
Mannobiosazone	m.p.	204-05°	203-06°	(33)

III.20.8 Examination of Fraction V and Identification of 6^2-α-Galactosyl Mannobiose

R_{Man} 0.08 and 0.17 in solvents (C) and (B) respectively. The sugar was recrystallised from 90% ethanol. The paper parti-

tion chromatography revealed only one spot, R_{glu} in solvent (G) 0.33 ; m.p. 226-28° and $[\alpha]_D^{32}$ + 98.6° (in water, C, 0.49 g per 100 ml of solution). It reduced Fehling's and Tollen's reagents.

The complete acid hydrolysis with 2% sulphuric acid, ~~and~~ neutralisation with barium carbonate and chromatographic examination showed the presence of galactose and mannose in the sugar. The quantitative estimation by the method of Hirst and Jones[47] showed that galactose and mannose constitute the oligosaccharide in the molar ratio of 1:2. The equivalent weight was found to be 262.8 by hypoiodite method[46].

The periodate oxidation studies revealed that one mole of of the oligosaccharide consumed 6.30 moles of metaperiodate and liberated 3.18 moles of formic acid. Partial acid hydrolysis revealed the presence of mannobiose and epimelibiose besides galactose and mannose.

From the above observation, the sugar was identified to be α-Galp-1 ⟶ 6-β-Manp-1 ⟶ 4-Manp. The observed data were found in close agreement with the reported values in literature as shown in Table - 13.

TABLE - 13

Constants	Found	Reported	References
m.p.	226-28°	220-29°	(72,92)
Optical rotation	$[\alpha]_D^{32}$ + 98.6°	$[\alpha]_D^{25}$ + 93.3° ⟶ + 98.6°	(72,92)
R_{Glu} in solvent (G)	0.33	0.32	(36)

III.29.9 Examination of Fraction VI and Identification of 3-O-α-Galactopyranosyl-D-Galactose

R_{Gal} = 0.60 in solvent (G). The sugar was recrystallised from methanol. $[\alpha]_D^{30}$ + 15.2° (in water, C, 1.2%).

Acid hydrolysis with 2% sulphuric acid followed by neutralisation with barium carbonate and paper chromatographic examination showed the presence of galactose units only. The equivalent weight was found to be 173.4 by hypoiodite method[46] which corresponded to a disaccharide of hexose units.

Periodate oxidation studies of the sugar revealed the consumption of 3.12 moles of sodium metaperiodate liberating 1.08 moles of formic acid per mole of the oligosaccharide.

It could not be hydrolysed with emulsin indicating the linkage between the galactose unit to be α- ~~at~~ ~~-position.~~

The above observation identified the oligosaccharide to be 3-O-α-galactopyranosyl-D-galactose. The identity was further confirmed by preparing its osazone, m.p. 238-37°, and acetate, m.p. 153-55°.

TABLE - 14

Sugar or derivative	Constant		Found	Reported	References
Galactobiose	Optical rotation	$[\alpha]_D^{30}$	+ 152°	$[\alpha]_D$ + 150°	(92)
Osazone	m.p.		235-37°	237-239°	(92)
Acetate	m.p.		153-55°	157-58°	(92)

III.23.10 Examination of Fraction VII and Identification of D-Galactose

R_G = 0.82 in solvent (G), R_{Man} = 0.62 and 0.80 in solvents (A) and (B) respectively. The sugar crystallised from aqueous methanol, $[\alpha]_D^{32}$ + 80.2° (in water, C, 1.0%). It was identified to be D-galactose by co-chromatography with an authentic sample.

III.23.11 Examination of Fraction VIII and Identification of D-Mannose

R_F = 0.12 in solvent (A) and R_G = 1.08 in solvent (G), $[\alpha]_D^{32}$ + 12.6° (in water, C, 2.0%). The sugar was identified to be D-mannose by co-chromatographic examination with an authentic sample.

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CHAPTER - IV

SUBJECT AND PLANNING

FROM THE NOVEL OF

RAJKUMAR CARDIA

IV.1 In the present Chapter chemical examination of a sterol and two flavonoids from the seeds of Daucus carota Linn., has been described.

Daucus carota Linn., commonly known as 'Gajar' (The Carrot), belongs to the family umbelliferae[1], is a hispid herb, 1 - 4 ft. high. Leaves 2-3 pinnae ; pinnae pinnatifid, segments narrow-lanceolate. Outer rays connivent in fruit; bracteoles many 3-fid and simple. Fruit 0.1 inch; bristles of secondary ridges long, glistening white, connate at base only, of the primary ridges small, subglochidiate, carpophore undivided.

The carrot is extensively grown within the area, as a cold weather crop. The plant is found wild in Europe, extends through west Asia, west-wards to Kashmir and along the Himalayan Ranges within the temperate zone, and cultivated throughout India.

The seeds are useful in diseases[2] of kidney and in dropsy, nervine tonic and given in to uterine pains. Antipolyneurite substances from carrot[3] may cure polyneuritis in pigeons in those cases where the disease has developed quickly (within 20 days).

IV.2 The details of research work reported in the literature on this plant is given in tabular form on the next page.

Plant	Parts	Constituents	References
1. Carrot	-	Antipolyneuratic substances	(1918)[3]
2. Carrot (Red & white) Asiatic)	-	Vitamin A content	(1934)[4]
3. Carrot	-	Rosducosterol	(1937)[5]
4. Carrot	-	Amino acid composition (Arginine, Histidine, Isoleucine, Leucine, Lysine, Methionine, Phenylamine, Threonine, Tryptophan and veline).	(1949)[6]
5. Carrot (In Poland)	-	Choline content	(1950)[7]
6. Carrot	-	Pentothonic acid	(1952)[8]
7. Carrot	-	Anthocyanins	(1957)[9]
8. Carrot	-	Lycopersene	(1965)[10]
9. Carrot	Roots	Carotoin	(1939)[11]
10. Carrot	Roots	Xanthophyll	(1948)[12]
11. Carrot	Roots	Amino acid composition (Alanine, Aspartic acid, Glutamic acid, Asparagine Cystine, Histidine, Isoleucine, Lucine, Methionine, Threonine, Tryptophan, Arginine, Glutamine, Glycine, Serine and Veline)	(1950)[13]
12. Carrot	Roots	Aldrin and Dieldrin	(1960)[14]
13. Carrot	Roots	Organic acid - Malic, Citric Isocitric and trace of Succinic and Fumaric acids.	(1962)[15]
14. Carrot	Leaves	α-Carotene	(1933)[16] (1956)[17]

(Continued)

Plant	Parts	Constituents	References
15.Carrot	Leaves	Aldrin and Dieldrin	(1960)[14]
16.Carrot	Leaves	Carnithine Carbamoyl transferase inhibitor	(1963)[18]
17.Carrot	Fruits	Vitamin C	(1956)[19]
18.Carrot	Fruits	Cyanidine	(1962)[20]
19.Carrot	Seeds	Disinfectants	(1953)[21]
20.Carrot	Phloem	Cellulose	(1961)[22]
21.Carrot	Green	Glycosidic bitter principle ethereal oil, wax-like petr. ether soluble fat and proto-alkaloids - (Pyralidine and Daucine).	(1951)[22a]
22.Carrot	Juice	Total sugars, Protein, Fats, Capectate, Total acidity ash, Ca, K, Na, P, Cl, Total Carotene β-Carotene Vitamin C.	(1963)[23]
23.Daucus carota	-	Ethereal oil.	(24, 25)
24.Daucus carota	-	Vitamin V_2 content	(1952)[26]
25.Daucus carota	-	β-Carotene content	(1952)[27]
26.Daucus carota	Roots	Fructose, Glucose, Sucrose	(1947)[28]
27.Daucus carota	Roots	Fibrillar lignin and Fibrillar pectin	(1971)[29]
28.Daucus carota	Leaves & berries	Vitamin C	(1946)[30]

(Continued)

Plant	Parts	Constituents	References
30. Daucus carota	Leaves	Lutedin-7-glucoside	(1960)[31]
31. Daucus carota	Leaves	Hydrocarbons, Alcohols, and Phytosterols	(1960)[31a]
32. Daucus carota	Flowers	Pigments - Kaempferol-3 glucoside, Kaempferol-3-diglucoside & apigenin	(1963)[32]
33. Daucus carota	Fruits	Ethereal oil composition (Carotol, Geranyl acetate and epoxidihydroxyeamyophyllin)	(1964)[33]
34. Daucus carota	Fruits	Volatile oil composition Free acids, consisting of Isobutyric and Palmitic acids and small amount of Aldehyde, ethers, terpenes, pinene and l-linolene	(34)

From the survey of literature it seems that the roots, leaves, flower, fruits, and seeds of the genus have been extensively examined for various plant products, but no work has been reported on the study of polysaccharide. On chemical examination a polysaccharide was isolated from this plant. But due to the paucity of the amount of polysaccharide , the entire study has not been possible. Since no study on flavonoid compounds too has been reported from the seeds, therefore, author became interested to study thoroughly the chemical constituents from the seeds of Daucus carota.

IV. 3- EXTRACTION AND ISOLATION OF STEROL AND FLAVONOIDS FROM THE SEEDS OF DAUCUS CAROTA

The seeds of Daucus carota were collected locally and identified for their authenticity in the Botany Department of D. V. Postgraduate College, Orai.

Dried and crushed seeds (2 kg) were defatted with petroleum ether (60 - 80°) in a soxhlet extractor. This extract was concentrated and allowed to stand for a few days, when a dirty white substance settled down. The deposit was filtered from the extract dissolved in benzene and adsorbed over a column of neutral alumina. The column was eluted with the mixture of petroleum ether - benzene (1:3). The eluate was concentrated to give a substance which was recrystallised from chloroform - methanol (9:1) as white flakes, compound (D), m.p. 138°. It gave characteristic Liebermann-Burchard test for sterols.

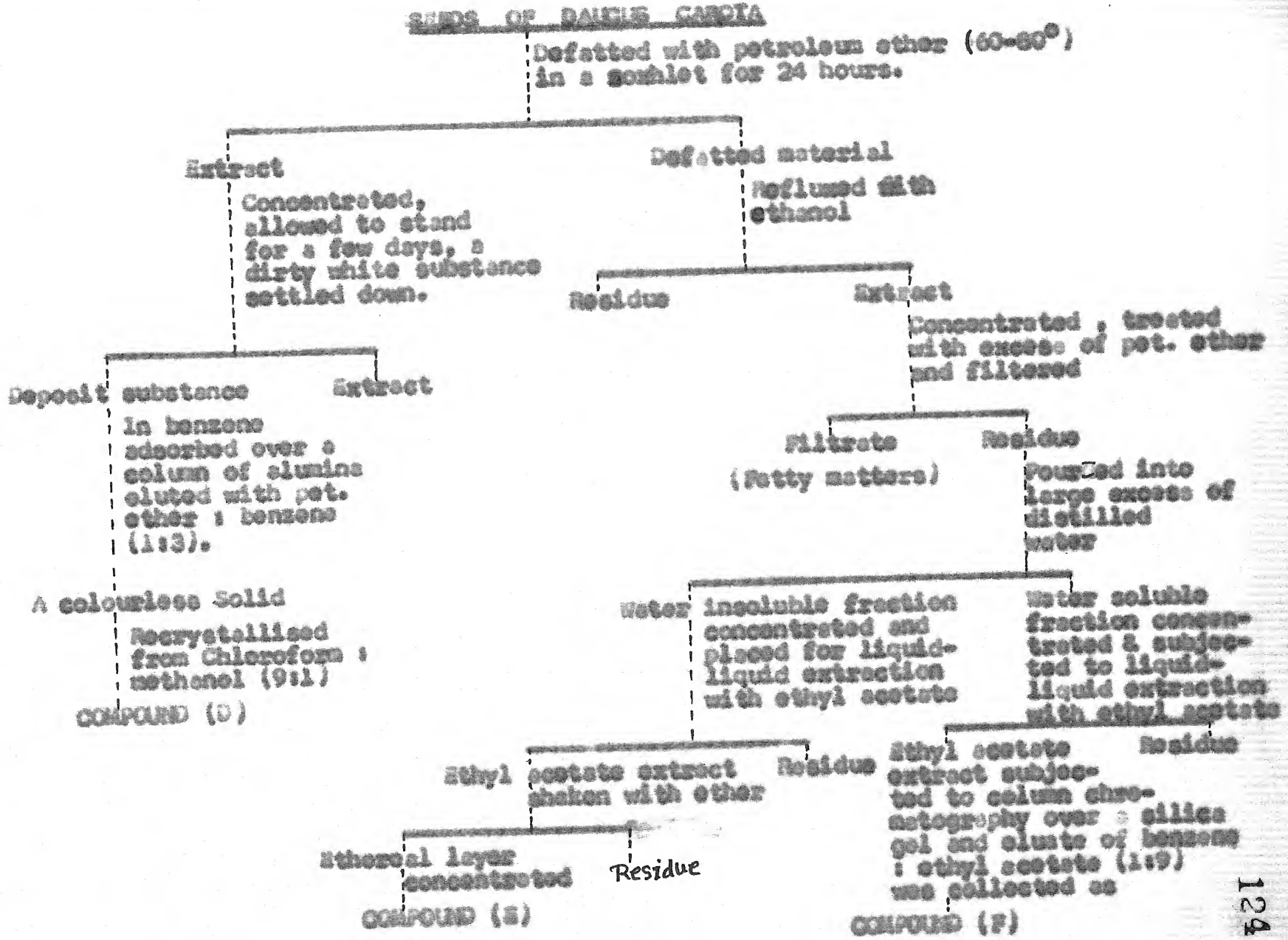
The defatted material was extracted with ethanol (95%) on a steam-bath in several lots. The total extract was concentrated at reduced pressure to a brown viscous mass. It was refluxed with petroleum ether (40 - 60°) to remove the fatty material and resulting residue , still viscous mass, was poured into a large excess of distilled water with vigorous stirring. The water soluble and insoluble fractions were separated and successively subjected to liquid - liquid extraction, using petroleum ether, benzene, ethyl acetate and acetone separately.

The ethyl acetate fraction of water insoluble part, was shaken several times with ether in a separating funnel. The

ethereal layer was separated and the solvent was evaporated to dryness where upon a light yellow substance was obtained. This on crystallisation from acetone - methanol (1:1) gave compound (E), m.p. 278°.

The acetone extracts of water soluble part was subjected to column chromatography over a silica gel G. The benzene - ethyl acetate (1:9) eluate of the column yielded a dark yellow coloured compound (F), m.p. 266-70°.

IV.4


SEEDS OF DALBERGIA SISSOO
Defatted with petroleum ether (60-80°) in a soxhlet for 24 hours.
Extract
Concentrated, allowed to stand for a few days, a dirty white substance settled down.
Defatted material
Refluxed with ethanol
Residue
Extract
Concentrated, treated with excess of pet. ether and filtered
Deposit substance
Extract
In benzene adsorbed over a column of alumina eluted with pet. ether : benzene (1:3).
Filtrate
(Fatty matters)
Residue
Poured into large excess of distilled water
A colourless Solid
Recrystallised from Chloroform : methanol (9:1)
COMPOUND (D)
Water insoluble fraction concentrated and placed for liquid-liquid extraction with ethyl acetate
Water soluble fraction concentrated & subjected to liquid-liquid extraction with ethyl acetate
Ethyl acetate extract shaken with ether
Residue
Ethyl acetate extract subjected to column chromatography over silica gel and eluate of benzene : ethyl acetate (1:9) was collected as
Residue
Ethereal layer concentrated
Residue
COMPOUND (E)
COMPOUND (F)

SECTION - A

IV.3 CHEMICAL STUDY OF COMPOUND (D)

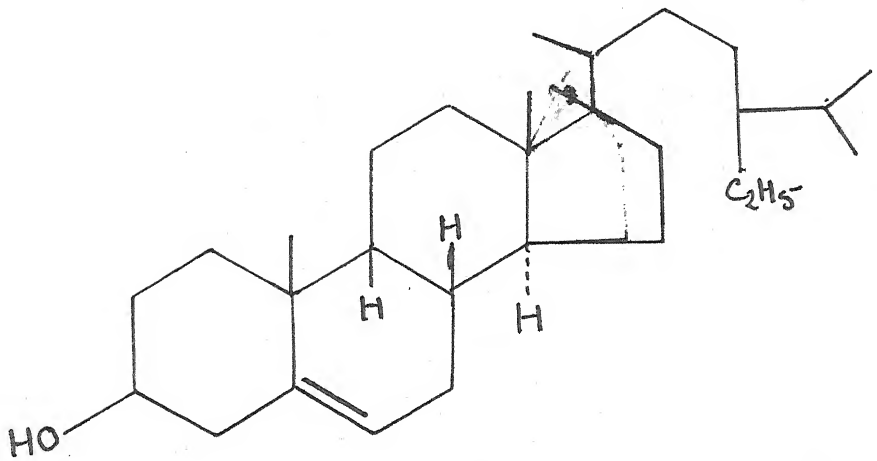
Compound (D), m.p. 130°. $[\alpha]_D^{28}$ - 36.6° (in chloroform), was isolated from the seeds of Daucus carota as described on page 122. The compound was found to have molecular formula, $C_{29}H_{50}O$. and soluble in petroleum ether, hexane, chloroform, ethyl acetate, ethanol and methanol.

The compound gave all colour reactions specific for sterol. i.e., Liebermann-Burchard reaction[35], Salkowski reaction[36], Tschugajew reaction[37] and Rohlenberg's reaction[38]. It also gave red colour with Noller's reagent[39]. These reactions are specific for steroids and terpenoids. Since the compound did not produce any colour by Brieskorne test[40], showing the absence of triterpenoids. From the molecular formula and colour reaction, it is evident that the compound (D) is a sterol. It also gave positive test with tetranitromethane[41], indicating the presence of olefinic bond in the molecule, which is further supported by the peaks at 845 and 805 cm^{-1} (Tri- substituted olefin) in its IR spectrum.

On acetylation a monoacetyl derivative, $C_{31}H_{52}O_2$, m.p. 126°. $[\alpha]_D^{28}$ - 39.4° (in chloroform) and on benzoylation a monobenzoyl derivative, $C_{36}H_{54}O_2$, m.p. 142°. $[\alpha]_D^{28}$ - 14.4° (in chloroform) were obtained. These results showed the presence of only one hydroxyl group in the compound. It also formed digitonide, m.p. 218°. The IR spectra of the compound gave a peak at 3400 cm^{-1} [42,43] characteristic of hydroxyl group which is further confirmed by the hydroxyl group proton signal in NMR spectra contained at δ 3.42(triplet).

The NMR spectrum of the compound reveals the presence of five methyl groups centered at 0.68 (s) for methyl group at C-13, 0.82 (t) for methyl group at C-29, 0.92 (d) for two methyl groups at C-25, 0.96 (d) for methyl group at C-20 and 1.12 (S) for methyl group at C-10.

On the basis of fore-going observations, the compound was identified to be β-sitosterol. The identity was confirmed by its mixed melting point, co-chromatography and the superimposition of its IR spectra over that an authentic sample of β-sitosterol[43]. Thus the compound (D) has been assigned the following structure.



β- Sitosterol (COMPOUND (D) .

(iii) Salkowski Reaction[36] - The chloroform solution of the compound on treatment with concentrated sulphuric acid[36], gave a yellow colour which changed to deep red.

(iv) It discharged the colour of potassium permanganate solution.

(v) Keller's Reaction[39] - The compound gave a deep red colour with a few drops of thionyl chloride (Prepared by adding 0.01% stannic chloride in pure thionyl chloride).

(vi) The compound was treated with concentrated hydrochloric acid, along with a few drops of ferric chloride and the resulting mixture evaporated to dryness. A red colour was produced when a few drops of water were added to the above mass.

(vii) A white precipiate was obtained when ethanolic solution of the compound was treated with ethanolic solution of digitonin.

ELEMENTAL ANALYSIS

Found	Calculated for $C_{29}H_{50}O$
C = 83.96%	C = 84.06%
H = 12.18%	H = 12.08%
molecular weight = 414 (Rast's method)	molecular weight = 414

IV.2 ACETYLATION OF THE COMPOUND

To the compound (40 mg), fused sodium acetate (1 g) and acetic anhydride (5 ml) were added and the whole reaction mixture was refluxed for nearly 10 hours over a sand-bath at 140°. The

action mixture was poured in ice cold water and precipitate so obtained was washed well with water, dried and recrystallised from chloroform - methanol (9:1) mixture, m.p. 125-27°.
$[\alpha]_D^{28}$ = 33.4° (in chloroform).

DETERMINATION OF ACETYL PERCENTAGE

The percentage of acetyl group in the acetylated derivative was determined by the method of Viamberger[44] as described by Belcher and Godivert[45].

Found	Calculated
C = 81.40%	C = 81.37%
H = 11.46%	H = 11.40%
Acetyl percentage = 11.02%	Acetyl percentage = 9.43%.

IV.10 BENZOYLATION OF THE COMPOUND

To the compound (20 mg), benzoyl chloride (2 ml) and four drops of pyridine were added in a Pyrex stoppered conical flask. The reaction mixture was kept for 20 hours and heated over a water-bath for six hours. The contents were cooled and poured in ice cold water containing 2% aqueous sodium bicarbonate. The yellow residue so obtained was washed well with 2% sodium bicarbonate and followed by water till it was free from the smell of benzoyl chloride. It was filtered, dried and recrystallised from chloroform - ethanol (8:2) mixture, m.p. 142°. $[\alpha]_D^{28}$ = 14.6°.

IV.11 PREPARATION OF DIGITOXIDA

The compound (30 mg) was dissolved in hot absolute ethanol

(5 ml) and treated with hot solution of digitonin (20 mg in 5 ml of absolute ethanol). The reaction mixture was heated over a water-bath for one hour, whereupon a white flocculent precipitate was obtained on cooling. It was washed well with ethanol, dried and recrystallised from hot ethanol as a flocculent white solid, m.p. 215-17° (lit. m.p. 215°).

IV.12 I.R. SPECTRUM OF COMPOUND (D)

The following peaks (cm^{-1}) in the IR spectrum (KBr) of the compound were observed by using Perkin-Elmer Infra-cord Spectrophotometer :

3620, 2942, 1642, 1468, 1379, 1252, 1198, 1129, 1068, 1030, 970, 845, 900, and 740.

IV.13 NMR SPECTRUM OF THE COMPOUND (D)

The NMR spectra of the compound (D) was taken on Varian A-60 Spectrometer, $CDCl_3$ as solvent and TMS as reference.

SIGNALS IN δ VALUE	ASSIGNMENT
0.68 (s)	Methyl group at C-13
0.82 (d) (J = 6 cps)	Methyl group at C-28
0.92 (d) (J = 2.40 cps)	2 Methyl group at C-25
0.96 (d)	Methyl group at C-20
1.12 (s)	Methyl group at C-10
1.00-2.40 (m)	Methyl groups
3.42 (t)	Methylenic hydroxyl group.

SECTION X - B

IV.14 CHEMICAL STUDY OF COMPOUND (E)

A light yellow coloured compound (E) , m.p. 276° was isolated from the ethanolic extract of the seeds of Daucus carota as described on page 122. The compound (E) having molecular formula, $C_{15}H_{10}O_6$, was shown to be a single entity by paper chromatography.

The ethanolic solution of the compound gave following colour reactions.

(i) It gave orange colour on treatment with magnesium powder and hydrochloric acid[46].

(ii) It gave an olive green colour with ethanolic ferric chloride[47].

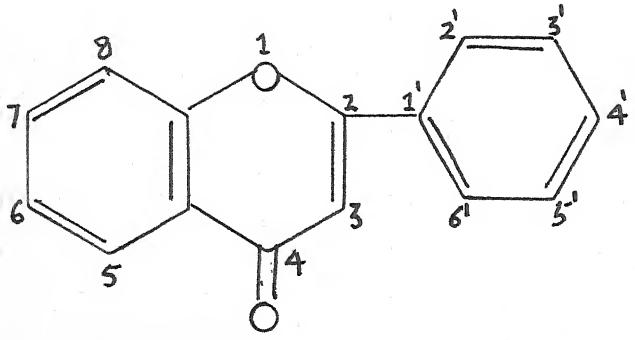
(iii) It produced deep yellow colour with liquid ammonia and showed flourescence under UV light[48].

(iv) A yellowish brown colour was obtained on treatment with sodium hydroxide solution.

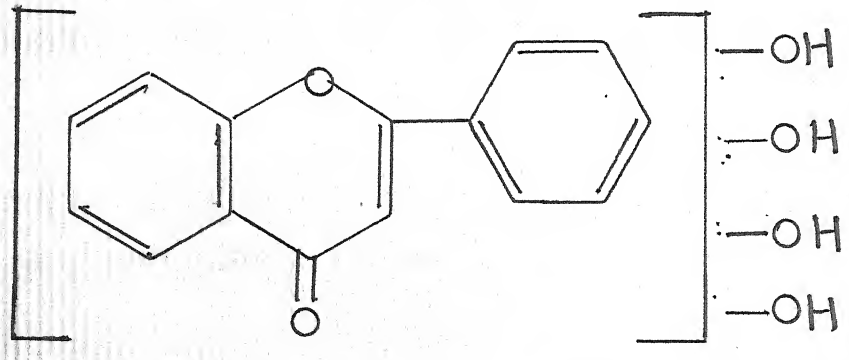
(v) It could not be reduced with sodium borohydride[49].

(vi) It did not give positive reaction with 2,4-dinitro phenyl hydrazine[50].

The above reactions suggest that the compound (E) is a flavone derivative having skeleton shown on next page.



The skeleton accounts only for $C_{15}H_{10}O_2$ which suggests that the remaining four oxygen atom may be present as four hydroxyl groups. The presence of this skeleton is also supported by the absorption maxima of the compound at 267 nm and 367 nm with an inflexion at 315 nm[40,41]. The compound formed a tetra acetate and tetramethyl ether on acetylation and methylation respectively, confirming the presence of four hydroxyl groups. Thus the compound may be represented as given below :



The relative positions of these hydroxyl groups have been assigned on the basis of various colour reactions, degradation and spectral studies of the compound. A free hydroxyl group at position -3 was shown by the following facts :

(i) A deep yellow colour was obtained when the methanolic solution of the aglycone was treated with zirconium oxychloride.

which in the presence of citric acid gave a white precipitate[52-54].

(ii) A deep yellow colour was obtained on addition of ethanolic boric acid and sodium acetate reagents to the ethanolic solution of the compound[55].

(iii) A red colour was produced on treatment with zinc dust and hydrochloric acid[56,57].

(iv) A bathochromic shift of 59 nm (λ_{max} from 367 nm to 426 nm) in the visible region of spectrum of the aglycone was recorded on addition of 1% ethanolic aluminium chloride to its ethanolic solution[58].

The compound gave pink colour with vanillin hydrochloric acid reagent, showing the presence of phloroglucinol unit in the structure, which corresponds to the presence of free hydroxyl groups at position -5 and at -7 [59,60]. The presence of free hydroxyl at position -5 is confirmed by the following facts :

(i) The compound produced an olive green colour with ethanolic ferric chloride[47] and a spot of this solution gave a yellow-violet fluorescence under UV light[61].

(ii) It gave a yellow colour, on treatment with a solution of boric acid and citric acid in acetone, which showed a yellowish green fluorescence[62,63].

(iii) The two separate spots of the compound on a filter paper produced bright fluorescence under UV light , when they were treated separately with ethanolic solutions of aluminium chloride and zirconium oxychloride[61,64].

(iv) It gave red colour[65] on treatment with Dimroth's reagent.

The presence of a free hydroxyl group at position -7 is confirmed by the fact that a bathochromic shift of 11 nm (λ_{max} from 267 nm to 278 nm) was observed on addition of fused sodium acetate to the ethanolic solution of the compound[52].

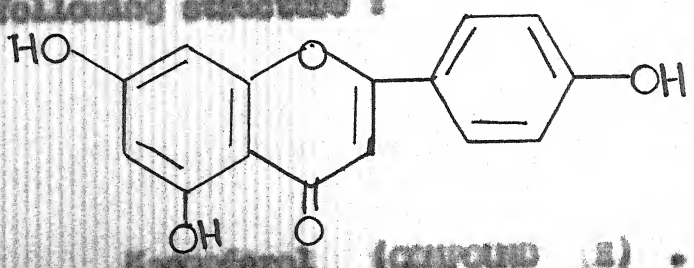
The remaining fourth hydroxyl group should occupy the position -4' in the ring B on the basis of following facts :

(i) A blue colour was produced on addition of sodium bicarbonate to the pink solution obtained by Shinoda reduction of the compound[66].

(ii) The methyl ether of the compound, on oxidation with neutral potassium permanganate gave anisic acid , m.p. 180° as one of the major oxidation products.

(iii) The visible region absorption maxima of the compound disappeared (λ_{max} from 367 nm to 341 nm) when 0.002M sodium ethoxide was added to its ethanolic solution, which showed a free hydroxyl at position -4' in conjugation with a free hydroxyl group at position -3 [67,68,69].

The above evidences indicated that the compound should have the following structure :



Kaempferol (COMPOUND 2) .

This represents the well known compound Kaempferol , which is supported by absorption maxima of the solution obtained after Shinoda reduction (λ_{max} 513 nm in methanol)[69] and IR peaks at 1665 cm^{-1} and 1615 cm^{-1} of the compound[70,71]. Finally the identity of the compound was confirmed by its mixed melting point and co-chromatography with an authentic sample.

EXPERIMENTAL.

IV.15 ISOLATION AND PURIFICATION

The compound (I), m.p. 278°, was isolated from the water insoluble fraction of ethanolic extract of the seeds of Daucus carota as described on page 122.

IV.16 HOMOGENEITY OF THE COMPOUND

The homogeneity of the compound (I) was tested on Whatman No.1 filter paper when a single spot was observed in each case using the following solvent systems :

		R_f
(i)	n-Butanol - acetic acid - water (4:1:5 v/v)	0.85
(ii)	Phenol saturated with water	0.60
(iii)	Acetic acid : water (60:40 v/v)	0.92

ELEMENTAL ANALYSIS OF THE COMPOUND

Found	Calculated for $C_{15}H_{10}O_6$
C = 62.70%	C = 62.93%
H = 3.62%	H = 3.49%
molecular weight = 286	molecular weight = 286

IV.17a ACETYLATION OF THE COMPOUND

The compound (40 mg) was acetylated with acetic anhydride (5.0 ml) and pyridine (3.0 ml). The reaction mixture was left overnight and poured in ice-cold water with constant stirring. It was filtered, washed well with water, dried and recrystallised from methanol to yield acetyl derivative, m.p. 118°.

DETERMINATION OF ACETYL PERCENTAGE

The acetyl percentage in the acetylated derivative was determined by the method of Viesenberger[44] as described on by Godbert and Belcher[45].

	Found	Calculated for $C_{15}H_8O_6(COCH_3)_4$
Acetyl group	36.96%	37.08%

IV.17b METHYLATION OF THE COMPOUND

The compound (2) (40 mg) was taken in dry acetone (20 ml) and was methylated with dimethyl sulphate (5 ml) and anhydrous potassium carbonate (1.0 g) by refluxing it on a water-bath for 20 hours. The reaction mixture was cooled, filtered and poured over crushed ice whereupon a yellowish mass was settled down. It was filtered, washed well and recrystallised from ethanol, m.p. 151° (R_f 0.32 in 15% acetic acid).

DETERMINATION OF METHOXYL PERCENTAGE

The methoxyl percentage in the methylated product was determined by the method of Belcher, Fildes and Nutten[72].

	Found	Calculated for $C_{18}H_6O_2(OCH_3)_4$
Methoxyl group	37.28%	36.22%

IV.18 POTASSIUM PERMANGANATE OXIDATION OF THE METHYL ETHER OF THE COMPOUND

The methylated compound (20 mg) was oxidised with neutral

potassium permanganate solution under reflux for four hours. The reaction mixture was cooled and the excess of manganese dioxide was destroyed by adding sodium bisulphite to it. The resulting solution was acidified with dilute hydrochloric acid, whereupon a white compound separated. It was filtered and crystallised from ethanol, m.p. 180°. It was identified to be anisic acid by its mixed melting point and co-chromatography with an authentic sample (R_f 0.36 in n-butanol saturated with ammonia ; spray = bromophenol blue solution).

IV.19 UV and VISIBLE SPECTRA

UV and visible spectra were recorded on Beckman model DU Spectrophotometer.

S.No.	Solution and Reagent	λ_{max} (nm)	Shift (nm)
(i)	E + Ethanol	267 , 367	- -
(ii)	E + Ethanol + $AlCl_3$	- , 426	- 59
(iii)	E + Ethanol + NaOAc	278 , -	11 -
(iv)	E + Ethanol + NaOEt	- , 341	- 26
(v)	E + Methanol + Mg + HCl	- , 313	- -

I.R. SPECTRA

Following prominent peaks (cm^{-1}) were observed in the IR spectrum of the compound.

3450, 3300, 1660, 1615, 1578, 1502, 1431, 1372, 1040, 825, and 795.

SECTION - C

IV.20 CHEMICAL STUDY OF THE COMPOUND (F)

Ethyl acetate extract of water soluble fraction of ethanolic extract of seeds of Cassia occidentalis afforded a compound (F), m.p. 268-70°, having molecular formula, $C_{27}H_{30}O_{15}$. It was isolated from the seeds as described on page 122, and was shown to be single entity by paper chromatography.

The compound gave the following colour reactions :

(i) It gave pink colour in Shinoda reduction[46], but did not give pink colour with hydrochloric acid only.

(ii) It gave an yellow orange colour with ethanolic ferric chloride[47].

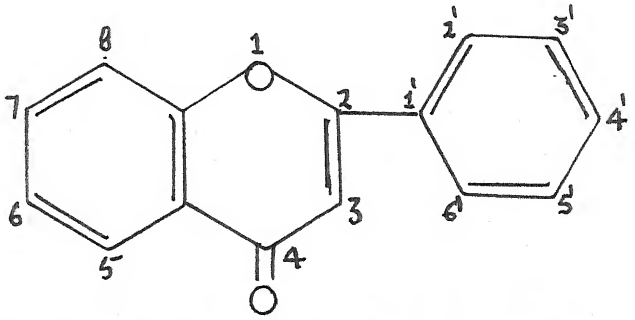
(iii) It produced yellow colour with liquid ammonia and showed yellow fluorescence in UV light[48].

(iv) A yellowish colour was obtained on treatment with sodium hydroxide solution, which was stable on heating[73].

(v) With concentrated sulphuric acid, it gave intense yellow colour with characteristic fluorescence[74,75].

(vi) No change in colour was observed on addition of vanillin hydrochloric acid reagent to the compound (F).

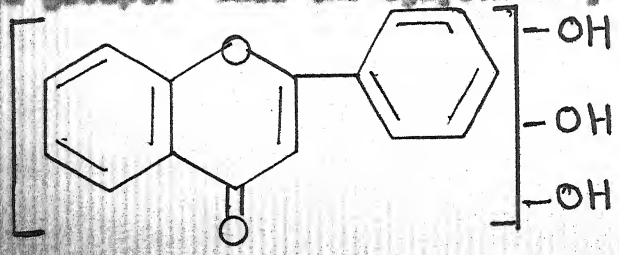
The above reactions suggest that the compound (F) is a flavone derivative possessing following skeleton



It gave positive Molisch's test indicating thereby the glycosidic nature of the compound. It is further supported by IR spectrum of the compound which exhibits the peaks at 1120 cm^{-1} and 1060 cm^{-1} The exact nature of the glycoside was indicated by the identification and characterisation of the aglycone and sugar moiety obtained on acid hydrolysis of the compound.

IV.21 AGLYCONE

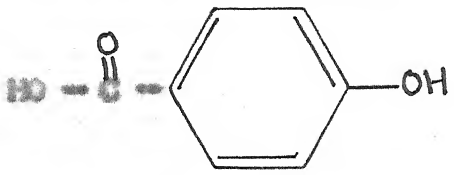
The pale yellow coloured aglycone, m.p. 349° has the molecular formula, $C_{15}H_{10}O_5$ and responded to colour reactions (1 – 6) described before. The presence of this skeleton is also supported by the absorption maxima of the compound at 269 nm and 336 nm. The skeleton accounts only for $C_{15}H_{10}O_2$, which suggests that the remaining three oxygen atoms may be present as three hydroxyl groups The compound formed triacetate and a trimethyl ether on acetylation and methylation respectively, confirming the presence of three hydroxyl groups. Thus the aglycone may be represented as below :



The relative positions of these hydroxyl groups have been assigned on the basis of various colour reactions, degradation and spectral studies of the aglycone.

The aglycone on oxidation with neutral potassium permanganate gave a compound identified as p-hydroxy benzoic acid.

Aglycone of compound (F) $\xrightarrow[\text{oxidation}]{\text{Potassium permanganate}}$



p-Hydroxy benzoic acid

This reaction shows that one hydroxyl group is present at position -4' of B ring of the compound. This was further confirmed by the following facts :

(i) When an excess of sodium bicarbonate was added to the solution resulting from Shinoda reduction of the compound, a blue colour[76,77] was obtained, showing the presence of free hydroxyl group at position -4'.

(ii) An ethanolic compound of the compound showed a bathochromic shift of 40 nm of Band I (from 336 to 376 nm) by the addition of fused sodium acetate, indicating the presence of hydroxyl groups at position -4' or -3 [52]. The possibility of hydroxyl group at position -3 was eliminated by the fact that the yellow colour given by the compound with aqueous sodium hydroxide was stable on heating[73].

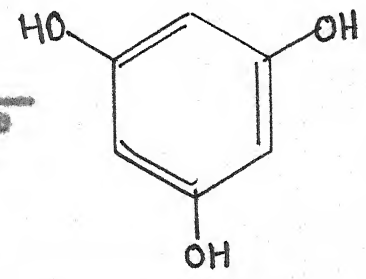
(iii) A bathochromic shift of 56 nm of Band I (from 336 nm to 392 nm) without a decrease in relative intensity was observed by the addition of sodium ethoxide to the methanolic solution of the compound. This shift is diagnostic[67,68] for the presence of free hydroxyl group at position -4'.

(iv) A single well defined peak (269 nm) of band II of the compound in ethanol also confirmed the presence of 4'-substituent in the B-ring[70].

The position of 4'-substituent in the ~~in the~~ B ring was further supported by NMR dates. The doublet 3-3.2δ is the chemical shift for C-3' and C-5' protons while the other one at down-field, 2.1 - 2.3δ is for C-2' and C-6' protons of B-ring. Hence the doublet for the C-3' and C-5' protons, shielded by the C-4' oxygen substitution.

The aglycone of the compound (F) on fusion with potassium hydroxide gave[79] a compound identified to be phloroglucinol. This degradation showed the presence of free hydroxyl groups at positions -5 and -7.

Aglycone of compound (F) $\xrightarrow{\text{50\% Aqueous Potassium hydroxide}}$



Phloroglucinol.

The presence of free hydroxyl group at position -5 was ~~further~~ supported by the following facts :

(i) The aglycone (F) gave an orange red colour with

Dimroth's reagent (acetyl pyroborate)[65].

(ii) The aglycone gave bright yellow colour with methanolic zirconium oxychloride showing the presence of free hydroxyl group at position -3 [80]. The colour did not change on addition of citric acid showing the absence of hydroxyl group at position -3 in the molecule[80].

(iii) When the aglycone of compound (F) in acetone was treated with a solution of boric acid and citric acid in acetone, a yellow colour with a yellowish green fluorescence developed. This shows the presence of methoxyl or hydroxyl group at position -5 [81].

(iv) An ethanolic solution of the aglycone gave green colour with the ethanolic ferric chloride[47].

(v) Bathochromic shifts of 46 nm in Band I (from 336 nm to 382 nm) and of 9 nm in Band II (from 269 nm to 278 nm) were observed by the addition of a few drops of ethanolic aluminium chloride to the ethanolic solution of the aglycone. This showed a free hydroxyl group at position -5 of the aglycone[82,83].

The presence of free hydroxyl group at position -7 of the aglycone of the compound (F) was supported by the following facts :

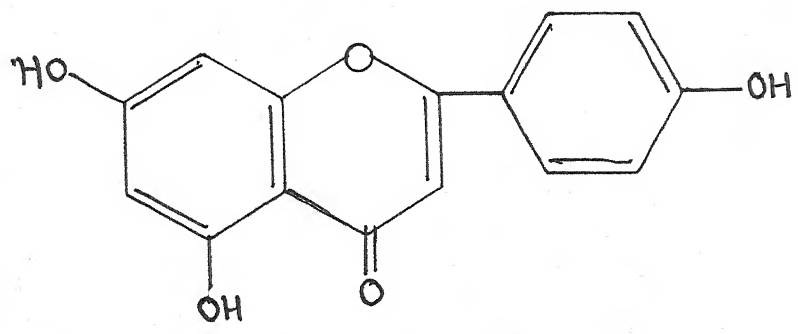
(i) Pink colour was given by the aglycone with vanillin hydrochloride reagent, indicating the presence of 5,7-dihydroxy grouping[60] in the molecule.

(ii) A bathochromic shift of 9 nm of Band II (from 269 nm to 278 nm) was observed on addition of a little fused sodium acetate

to the ethanolic solution of the aglycone, confirming the presence of free hydroxyl group at position -7.[32] The aglycone also did not give any precipitate with neutral lead acetate[61] showing the absence of ortho-dihydroxy grouping.

The supporting NMR data of 5,7-dihydroxy grouping in the aglycone of compound (F) showed ad doublet at 3.9δ and another doublet at 3.5δ which are indicative of proton at C-6 and C-8 in the ring A. It has been observed that flavones which contain 5,7-dihydroxy grouping give rise to doublets (J = 2.5 cps) in the range 3.5 to 4.0δ . A sharp singlet observed at 3.7δ confirms the proton H-3.

Hence , on the basis of above observations, the aglycone of compound (F) has been assigned the following structure 4',5,7-trihydroxy flavone (Apigenin).



IV.22 IDENTIFICATION OF SUGARS

Paper chromatograph of the sugar solution using n-butanol - acetic acid - water (4:1:5 v/v) system revealed two spots with R_f values 0.16 and 0.20 respectively, suggesting the presence of D-galactose and D-mannose. The identity of the sugars was confirmed by co-chromatography with an authentic samples of the sugars.

IV.23 POSITION OF GLYCOSIDIC LINKAGE

The position of glycosidic linkage in the glycoside was determined by direct comparison of its physical and chemical properties with that of its aglycone.

(i) The glycoside did not respond to positive colour reaction with vanillin hydrochloric acid reagent whereas the aglycone indicating that the position -7 is involved in the glycosidic linkage.

(ii) The glycoside did not give any shift in Band II with fused sodium acetate whereas aglycone of the compound gave a bathochromic shift of 9 nm of Band II (from 269 nm to 278 nm), confirming the presence of a free hydroxyl group at position -7 in the aglycone and absence of it in the glycoside.

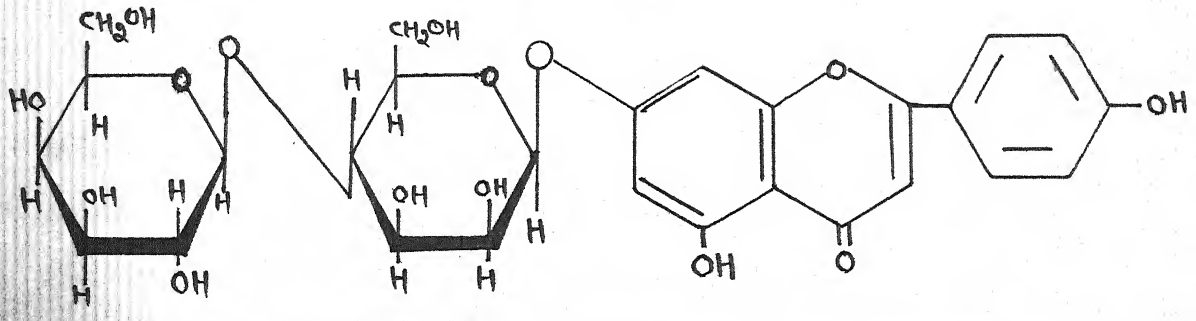
(iii) The glycoside as well as the aglycone both gave positive colour test on addition of sodium bicarbonate to their respective Shinoda's reduction products. This indicates the absence of glycosidic linkage at position -4' in the glycoside.

Thus , it is only the position -7 in the aglycone at which both the sugars, D-galactose and D-rhamnose are attached. The periodate oxidation studies of the glycoside showed the consumption of 3.16 moles of periodate with the liberation of 1.2 moles of formic acid per mole of the glycoside. It suggests that only one unit of each, galactose and rhamnose is present in the molecule which corresponded to the molecular formula , $C_{27}H_{30}O_{15}$ of the glycoside. The periodate oxidation studies also show that both the sugars are present in pyranose

form and are mutually linked through 1 → 4 linkage in the disaccharide. On partial acid hydrolysis of the glycoside by refluxing with 2% sulphuric acid an examined at different intervals by paper chromatography : galactose showed its appearance within one hour indicating that galactose occupies the terminal position. After two and a half hours of hydrolysis mannose appeared. The glycoside dissolved in benzol and hydrolysed with formic acid[84] for half an hour. The aqueous hydrolysate gave a single spot by paper chromatography. The R_f value of this entity was not found to be identical with the R_f value of various monosaccharides in different solvent systems[85]. Further hydrolysis of this hydrolysate with 7.0% H_2SO_4 gave galactose and mannose.

The completely methylated glycoside, on acid hydrolysis, gave 2,3,4,6-tetra-O-methyl-D-galactose and 2,3,6-tri-O-methyl-D-mannose which were identified by their R_{TMG}. It suggests that C_1 of mannose is involved in the glycosidic formation with the aglycone. Finally, the glycoside was completely hydrolysed with emulsin. This shows the presence of β-linkages between the galactose and mannose and mannose and aglycone.

The above all evidences suggest that the compound (F) is Apigenin-7-O-β-D-galactopyranosyl-(1 → 4)-O-β-D-mannopyranoside and may be represented as below :



Apigenin-7-galactomannoside Compound (F) .

EXPERIMENTAL.

IV.24 ISOLATION AND PURIFICATION.

The compound (F), m.p. 260-70° was isolated from the seeds of Daucus carota as described on page 122.

IV.25 HOMOGENEITY OF THE COMPOUND

The homogeneity of the compound was checked by paper chromatography on Whatman No.1 filter paper using following solvent systems :

(i) n-Butanol - acetic acid - water (4:1:5 ~~30:3:10~~ v/v).

(ii) Phenol saturated with water.

(iii) Acetic acid : hydrochloric acid : water (30:3:10 v/v).

In each case a single spot was obtained.

ELEMENTAL ANALYSIS

Found	Calculated for $C_{27}H_{30}O_{15}$
C = 54.6%	C = 54.5 4%
H = 5.10%	H = 5.08%.
Molecular weight = 594	Molecular weight = 594.

The glycoside (400 mg) was hydrolysed with 7% ethanolic sulphuric acid (50 ml) on a water-bath for 10 hours. The hydrolysate was cooled, solvent distilled off, diluted with water and filtered. The precipitate was dried in vacuum, crystallised from ethyl acetate - petroleum ether (7:3) mixture and finally recrystallised from methanol to yield a pale yellow coloured compound

(aglycone), m.p. 349°. The filtrate obtained after removal of the aglycone was neutralised with barium carbonate, filtered and concentrated under reduced pressure to a syrupy mass.

IV.26 EXAMINATION OF AGLYCONE

It was soluble in ethanol, methanol, acetone, pyridine and insoluble in petroleum ether, benzene and water. It gave all positive tests, characteristics of flavonoids, as described on page for the study of aglycone.

IV.27 CHROMATOGRAPHY OF AGLYCONE

The purity of the aglycone was checked on Whatman No.1 filter paper when a single spot was observed in each case using following solvent systems :

		R_f
(i)	n-Butanol - acetic acid - water (4:1:5 v/v)	0.88.
(ii)	Phenol saturated with water	0.93.
(iii)	m-Cresol - acetic acid - water (50:2:48 v/v)	0.87.

ELEMENTAL ANALYSIS OF THE AGLYCONE

Found	Calculated for $C_{15}H_{10}O_5$
C = 66.72%	C = 66.6%.
H = 3.80%	H = 3.70%.

IV.28 ACETYLATION OF AGLYCONE

The aglycone (40 mg) was acetylated with acetic anhydride (20 ml) and pyridine (3.0 ml). The reaction mixture was left

overnight and poured in ice-cold water with constant stirring. It was filtered, washed well with water, dried and recrystallised from methanol to yield acetyl derivative , m.p. 186-88°.

DETERMINATION OF ACETYL PERCENTAGE

The acetyl percentage in the acetylated derivative was determined by the method of Wiesenberger[44] as described by Godbert and Belcher[45].

Found	Calculated for $C_{19}H_{29}O_3(OCH_3)_3$
Acetyl group = 32.65%	= 32.57%.

IV.29 METHYLATION OF AGLYCONE

The aglycone (40 mg) was taken in dry acetone (20 ml) and was methylated with dimethyl sulphate (5 ml) and anhydrous potassium carbonate (1.0 g) by refluxing it on a water-bath for 20 hours. The reaction mixture was cooled , filtered and poured over crushed ice whereupon a yellowish mass settled down. It was filtered , washed well and recrystallised from ethanol, m.p. 186-87°.

DETERMINATION OF METHOXYL PERCENTAGE

The methoxyl percentage in the methylated aglycone was determined by the method of Belcher, Gibbs and Sutton[72].

Found	Calculated for $C_{19}H_{29}O_2(OCH_3)_3$
Methoxyl group = 30.0%	= 29.83%.

IV.30 POTASSIUM PERMANGANATE OXIDATION OF THE METHYL ESTER

The methylated glycoso (25 mg) was oxidised with neutral potassium permanganate solution under reflux for four hours. The reaction mixture was cooled and the excess of manganese dioxide was destroyed by adding sodium bisulphite to it. The resulting solution was acidified with dilute hydrochloric acid, whereupon a white compound separated. It was filtered and recrystallised from ethanol, m.p. 179°. It was identified to be mucic acid by its mixed melting point and co-chromatography with an authentic sample. (R_g 0.36 in n-butanol saturated with ammonia; spray bromophenol blue solution).

IV. 31. IDENTIFICATION OF SUGARS

The syrup obtained after the hydrolysis of the glycoside was examined paper chromatographically using n-butanol - acetic acid - water (4:1:5 v/v) as ascending solvent system. The developed chromatogram was air-dried, sprayed with aniline hydrogen phthalate and on heating at 120° for 10 minutes, two spots, R_g values 0.16 and 0.20 were observed, which corresponded to D-galactose and D-mannose respectively.

The identity of sugars was confirmed by co-chromatography with the authentic samples.

IV.32 METHYLATION OF THE GLYCOSIDE

The glycoside (50 mg) was methylated as described in case of the aglycone. The completely methylated derivative was crystallised from methanol.

IV.32.1 HYDROLYSIS OF METHYLATED GLYCOSIDE

The methylated glycoside (30 mg) was hydrolysed with 2% methanolic sulphuric acid (20 ml) on a water-bath for 4 hours under reflux. The reaction mixture was cooled, concentrated under reduced pressure, and poured in distilled water. The precipitate was filtered, washed well and recrystallised from methanol. The filtrate was neutralised with barium carbonate and concentrated under reduced pressure to a light yellow coloured syrup.

IV.32.2 IDENTIFICATION OF METHYLATED SUGARS

The syrup of the methylated sugars obtained as above was chromatographed on whatman No.1 filter paper using n-butanol - ethanol - water (5:1:4 v/v) as irrigating solvent system. The developed chromatogram was air-dried, sprayed with aniline hydrogen phthalate and heated to 120° for 10 minutes, whereupon two spots were obtained. The R_{TMG} values (TMG = 2,3,4,6-tetra-O-methyl-D-glucose) of the spots were found to be 0.60 and 0.90 which corresponded to 2,3,6-tri-O-methyl-D-galactose mannose and 2,3,4,6-tetra-O-methyl-D-galactose respectively. The identity of the sugars was confirmed by their co-chromatography with the authentic sample.

IV.33 PARTIAL HYDROLYSIS OF THE GLYCOSIDE

The glycoside (25 mg) was hydrolysed by refluxing with 2% sulphuric acid and hydrolysate was examined at different intervals by paper chromatography. Galactose showed its appearance after one hour. After two and a half hours of hydrolysis mannose could be detected.

be detected.

IV.24 HYDROLYSIS OF THE GLYCOSIDE WITH FORMIC ACID[84]

The glycoside (20 mg) was dissolved in boiling cyclohexanol (10 ml.) and hydrolysed with formic acid (7%, 8 ml) by refluxing on a water-bath for half an hour. The aqueous hydrolysate gave a single spot by paper chromatography. The R_f value of this entity was not found to be identical with the R_f value of various monosaccharides in different solvent systems[76]. Further hydrolysis of the hydrolysate with 7% H_2SO_4 gave galactose and mannose.

IV.25 PERIODATE OXIDATION OF THE COMPOUND

The glycoside (20 mg) was dissolved in a mixture of ethanol (25 ml) and distilled water (25 ml) and 0.25 M sodium metaperiodate (25 ml) was added to it. The solution was made upto 100 ml with ethanol and allowed to stand for 48 hours. The periodate consumed and the formic acid liberated were estimated by the titrimetric method of Jones et al.[75] The results are given below :-

Molecular weight of the compound	= 594
For 15 ml aliquote of the reaction mixture 0.01N sodium hydroxide consumed	= 0.38 ml
0.01N Hypo consumed	= 3.2 ml
For each mole of the glycoside	
Moles of formic acid liberated	= 1.2
Moles of periodate consumed	= 3.16

IV.36 ENZYMIC HYDROLYSIS

The glycoside (20 mg) was dissolved in aqueous ethanol and emulsin solution (25 ml), prepared from almonds[86] was added to it and the solution was kept at room temperature for four days. The hydrolysate after extraction with ethyl acetate, was concentrated to a syrup. The paper chromatography of the syrup in n-butanol - acetic acid - water (4:1:5 v/v) revealed the presence of two spots, R_g 0.16 and 0.20, corresponding to galactose and mannose respectively.

IV.37 UV AND VISIBLE SPECTRA OF THE COMPOUND (F)

UV and visible spectra were recorded on Beckman Model DU Spectrophotometer.

Solution and reagent	λ_{max} (nm)	Shift
(A) GLYCOSIDE		
(i) A + Ethanol	268 , 333	- -
(ii) A + Ethanol + NaOAc	268 , 336	- 33
(iii) A + Ethanol + $AlCl_3$	278 , 378	10 , 45
(iv) A + Ethanol + NaOEt	268 , 385	- 52
(B) AGLYCONE		
(i) B + Ethanol	269 , 336	- -
(ii) B + Ethanol + $NaOAc$	278 , 376	9 , 40
(iii) B + Ethanol + $AlCl_3$	275 , 382	9 , 46
(iv) B + Ethanol + NaOEt	277 , 392	8 , 56

IV.38 IR SPECTRUM OF COMPOUND (F)

Following prominent peaks (cm^{-1}) were observed in the IR spectrum of the aglycone :

3442, 3389, 1660, 1625, 1590, 1580, 1395, 1205, 1120, 1060, 842, and 710.

IV.39 NMR SPECTRUM OF AGLYCONE OF COMPOUND (F)

NMR Spectra was recorded on Varian A-60 instrument using TMS as the reference and the shifts are quoted in δ values :

SIGNALS IN δ VALUE	ASSIGNMENT
2.1 - 2.3δ (d)	Protons at C-2' and C-6'
3 - 3.2δ (d)	Protons at C-3' and C-5'
3.5δ (d)	Protons at C-8
4.27δ (s)	Proton at C-3
3.9δ (d)	Proton at C-6

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CHAPTER - V

CHEMICAL EXAMINATION OF WATER SOLUBLE MONOSACCHARIDES FROM THE FLOWERS OF ADHATODA VASICA NEES

Ved. The present Chapter deals with the isolation and identification of water soluble monosaccharides from the flowers of Linum usitatissimum, Linn., commonly known as Alsi, Tisi, Sani (Flax or Linseed) belonging to the family Linaceae[1].

Linum usitatissimum is a annual herb, stem, 2-4 ft., erect, usually corymbosely branched above. Leaves linear or lanceolate, without stipular glands, sub-3-nerved. Flower in broad cymes, blue or sometimes white, 1 inch across. Sepals ovate, acuminate, 3-nerved eglandular, margins white, ciliate or not. Stigmas linear - clavate. Capsule hardly exceeding the sepals ; edges of valves ciliate.

The plant is cultivated throughout India upto 6,000 ft especially in the Benares Division, in Bundelkhand and Sub-Himalayan tract.

The plant is of high medicinal value[2] Dried ripe seeds used as demule and in form of poultice, as poultice useful for gouty and rheum, swelling, used internally for irritation of genito -urinary system. Flowers used in hervine and cardiac tonic. Oils mixed with lime water used as application to burns.

From the survey of literature of Plant, the research work has been done till now shown in tabular form on the next page of this chapter.

Plant species	Parts	Constituents	References
1. Flax	-	Spinnable fibers	(1930)[3]
2. Flax	-	Textile fibers	(1933)[4]
3. Flax	-	Degradation and molecular size of flax pectin	(1940)[5]
4. Flax	Plants	Liberation of Plant fibers	(1936)[6]
5. Flax	Plants	Chemical composition plant fibers	(1969)[7]
6. Flax	Fibers	Viscose, artificial cotton and artificial wool	(1941)[8]
7. Flax	Fibers	Flavonoids, quercetin, Kaempferol, apigenin and genistein glycosides.	(1970)[9]
8. Flax	Stems	Separation of flax fibers	(1936)[10]
9. Flax	Stems	Pectin substance Pectin A and Pectin B	(1943)[11] (1943)[12]
10. Flax	Stalks	Pectin A and Pectin B	(1941)[13]
11. Flax	Seeds	Structure of aldobiomic acid from flax seed mucilage	(1939)[14]
12. Flax	Seeds	Mucilage, and fraction of mucilage	(1937)[15]
13. Flax	Seeds	Lignins	(1961)[16]
14. Flax	Seeds	Hydrolytic studies of mucilage, Galacto uronate	(1961)[17]

(Continued)

Plant species	Parts	Constituents	References
15. Flax	Seeds	Properties of mucilage, characteristics of some polysaccharide	
16. Flax	Seeds	Four polysaccharides from linseed mucilage	(1970)[18]
17. Flax	Seeds	Linamarin	(1952)[19]
18. Flax	Seeds	Fat content	(1963)[20]
19. Flax	Seeds	Free fatty acids	(1967)[21]
20. Flax	Seeds	Phosphatide content	(1968)[22]
21. Flax	Cotyledons	Six glycoflavone C-glycosids	(1969)[24]

From the study of chemical literature it seems that a countable work has been done on the seed mucilage and polysaccharide, and various other parts of the plant have been also investigated. But no work has been done on the flower of the plant. Due to its medicinal importance it was consider worthwhile to study the flowers of the plant.

V.2 EXTRACTION OF WATER SOLUBLE MUCOPOLYSACCHARIDES FROM THE FLOWERS OF LINUM USITATISSIMUM.

The flowers of *Linum usitatissimum* were collected locally and botanically identified in the Botany Department of D. V. Postgraduate College, Orai.

The crushed flowers were defatted with petroleum ether (60 - 80°) and extracted with cold distilled water. The whole lot was concentrated to a syrup which was examined for monosaccharides by paper chromatography and column chromatography.

The two sheets of paper were prepared and developed in Solvents (A) and (B). On spraying with aniline hydrogen phthalate each of the chromatogram showed the presence of five spots. The R_f and R_G values of these sugars corresponded to D-galactose, D-glucose, L-arabinose, D-xylose and L-rhamnose respectively. These sugars were further confirmed by co-chromatography with their authentic samples and on cellulose column, the chromatogram of above syrup was made which was run with the solvent (A) into different fractions. By checking each eluate on paper chromatography, total five fractions I, II, III, IV, and V were separated. The identity of each fractionw was tested by paper chromatography on Whatman No.1 filter paper with their authentic samples. The further identity of each was confirmed by their m.p., m.mp. , specific rotation and by preparing their derivatives.

M.2.1 Fraction I, was crystallised from aqueous ethanol, m.p. 93-94°. $[\alpha]_D^{37}$ + 8.6° (in water, C, 1%) gave pinkish colour with p-anisidine phosphate. It was identified to be L-rhamnose. Further identity was confirmed by preparing its p-anisidine phosphate derivative.

M.2.2 Fraction II, was crystallised from absolute ethanol, m.p. 143-44°. $[\alpha]_D^{30}$ + 17.5° (in ethanol water, C, 1.14%). This fraction was identified as D-xylose, and further confirmed by preparing xylosazone derivative.

Y.2.3 Fraction III also crystallised from aqueous ethanol, m.p. 156°. $[\alpha]_D^{29}$ + 101° (in water, C, 1%). From the above observations, the sugar was identified to be L-arabinose. Its identity was confirmed preparing L-arabinose phenyl hydrazone derivative.

Y.2.4 Fraction IV, crystallised from aqueous methanol, m.p. 146° , $[\alpha]_D^{21}$ + 53°. On the basis of the above results the sugar was identified to be D-glucose.

Y.2.5 Fraction V, crystallised from methanol, m.p. 165-68°. $[\alpha]_D^{29}$ + 80.8° (in water, C, 1%). All the above results indicated that the sugar was D-galactose. The identity was further confirmed by preparing its N-p-nitrophenyl-D-galactosylamine derivative.

EXPERIMENTAL

V.2 EXTRACTION OF SUGARS

Botanically identified, crushed flowers of *Linum usitatissimum* were defatted with petroleum ether (60 - 80°). The defatted flowers (200 g) were extracted with cold distilled water, for 12 hours and subsequently the solution was filtered. The insoluble portion was extracted with cold distilled water and filtered again. The two filtrates were combined and evaporated to a small volume in vacuum. This syrup was examined for monosaccharides by paper and column chromatography and preparing the derivatives of different monosaccharides.

V.2.1 PAPER CHROMATOGRAPHY

The following solvents systems were used for chromatography :

(A) n-Butanol - ethanol - water (4:1:5)[27,28].
(B) n-Butanol - acetic acid - water (4:1:5)[27].
(C) Ethyl acetate - acetic acid - water (3:1:3)[29].

Spray Reagent

Aniline Hydrogen Phthalate[30] - The reagent was prepared by adding aniline (0.93 g) and phthalic acid (1.66 g) to water saturated n-butanol (100 ml). Aldohexoses give brown colour and aldopentoses give bright red colour with this reagent.

V.2.2 Technique of PAPER CHROMATOGRAPHY

The spots of syrup were applied on two sheets of whatman

No.1 filter paper. The paper were developed separately in solvents (A) and (B) or by descending unidimensional technique. The chromatograms were air dried and sprayed with aniline hydrogen phthalate. On heating them in an oven at 120°, each chromatogram showed five spots. The R_g and R_g values of the five spots corresponded to D-galactose, D-glucose, L-arabinose, D-xylose and L-rhamnose as given in the following Table - 2.

TABLE - 2

Sugar identified	Solvent (A)		Solvent (B)	
	R_G found	R_G given[27,28]	R_g found	R_g given[27]
D-Galactose	0.06	0.07	0.15	0.16
D-Glucose	0.09	0.09	0.18	0.18
L-arabinose	0.11	0.12	0.20	0.21
D-xylose	0.16	0.15	0.27	0.28
L-rhamnose	0.29	0.30	0.36	0.37

G = 2,3,4,6-Tetra-O-methyl-D-glucose.

The identity of the five sugars was further confirmed by co-chromatography with authentic sample of the sugars in the same solvents.

V.A COLUMN CHROMATOGRAPHY

A large amount of above syrup was dissolved in a small amount of aqueous methanol (1:1) and adsorbed over a column of cellulose (2 x 35 cms). The column was left over-night and the separation was effected with Solvent (A). Fractions amounting

to 25 ml were collected and checked by paper chromatography with the authentic samples of different sugars.

I. L-Rhamnose

Fractions I - VI containing same sugar were combined together and concentrated to give L-rhamnose. It was crystallised from aqueous ethanol and gave crystals of α-L-rhamnose hydrate. Its m.p. and m.m.p. with an authentic specimen was found to be 93-94°. $[\alpha]_D^{37}$ + 8.6° (in water, C, 1%). Lit.[31,32,34].

It gave pinkish colour with p-anisidine phosphate.

DERIVATIVE

p-Anisidine Phosphate - It was prepared by dissolving p-anisidine (0.1 g) in phosphoric acid (4 ml, sp. gr. 1.75) and diluting the solution with ethanol (100 ml). The precipitated p-anisidine phosphate was filtered out , dissolving in minimum amount of water and mixed with ethanol (100 ml). This solution was acidified with phosphoric acid (2 ml; sp. gr. 1.75) and mixed with the first solution.

II. D-Xylose

Fraction 8-14 were mixed and concentrated to give D-xylose. It was recrystallised with absolute ethanol, m.p. 143-44°, not depressed by mixing with an authentic sample of D-xylose. $[\alpha]_D^{30}$ + 17.5° (in water, C, 1.14%).

DERIVATIVE

Xylosazone - In a test tube xylose (150 mg), phenyl hydra-

zine hydrochloride (300 mg) and sodium acetate (200 mg), dissolved in water (7 ml) and heated on a boiling water-bath for 30 minutes. Precipitate of the osazone started appearing after 7 minutes. The flocculent precipitate was separated with water and recrystallised from 90% ethanol, m.p. 160-61° equal to the the authentic sample.

XII. L-Arabinose

Fraction 19-21 containing same sugar, were combined together and concentrated to give L-arabinose. It was crystallised from aqueous ethanol, m.p. and m.m.p. with an authentic sample was 156°. $[\alpha]_D^{29}$ + 101° (in water, C, 1%). Lit.[31,32,34].

DERIVATIVE

L-Arabinose Phenyl Hydrazone - The sugar (200 mg), phenyl hydrazine hydrochloride (400 mg), crystalline sodium acetate (600 mg) and water (6 ml) were heated in a loosely stoppered test tube on a water-bath for 30 minutes. The tube was then cooled to room temperature, the crystalline osazone filtered out, washed with distilled water and recrystallised from aqueous ethanol, m.p. 163-64°, Lit.[36,37].

IV. D-Glucose

Fraction 23-27 were mixed and concentrated to give D-glucose. It was recrystallised from aqueous methanol, m.p. 146°. $[\alpha]_D^{21}$ + 53° , Lit.[31,32].

DERIVATIVE

Phenyl-D-Glucosazone - Sugar (40 mg), sodium acetate (40 mg) , 3 drops of phenyl hydrazine, 1 ml of water and 3 drops of glacial acetic acid were mixed. After boiling the whole contents for 30 minutes, 2 ml of water was added and cooled, washed with water. The crystals of phenyl-D-glucosazone were obtained and recrystallised from dilute ethanol into needle form. This derivative has , m.p. 205°.

V. D-Galactose.

Fraction 30-38 containing same sugar were combined together and concentrated to give D-galactose. It was recrystallised from aqueous methanol. Its m.p. 165-66°, $[\alpha]_D^{29}$ + 80.6°, (in water, C, 1%) lit.[31,32], m.p. 166-68°, $[\alpha]_D^{20}$ + 80.2° (in water, C, 1%)[33,34].

DERIVATIVE

N-p-Nitrophenyl-D-galactosylamine - In a micro test tube were taken galactose (50 mg), p-nitroaniline (50 mg), 1 drop of glacial acetic acids and 4 drops of methanol-water (8:1 v/v). The mixture was boiled for 8 minutes and kept overnight in a refrigerator. The crystalline product was filtered , washed with cold ethanol, after recrystallisation from methanol, the m.p. of the derivative was found to be 218-19°, lit.[35] , m.p. 219°.

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